FORM PTO-1390 (Rev. 1-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER J&J-1673

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/486757

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/AU98/00705	28 August 1998 (28.08.98)	29 August 1997 (29.08.97)

TITLE OF INVENTION

CYTOCHROME P450 REDUCTASES FROM POPPY PLANTS

APPLICANT(S) FOR DO/EO/US :

Kutchan, Toni M., Zenk, Meinhart H., Atkins, David G., and Fist, Anthony J.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) (Unexecuted)

10. ☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
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17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... $1070.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....$930.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.455(a)(2)) paid to USPTO..... $790.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... $720.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... . ... $98.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 930.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	62 - 20 =	42	x $22.00	$924.00
Independent claims	7 - 3 =	4	x $82.00	$328.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$
TOTAL OF ABOVE CALCULATIONS =				$2182.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).			+	$
SUBTOTAL =				$2182.00
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) .				$
TOTAL NATIONAL FEE =				$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	$
TOTAL FEES ENCLOSED =				$
			Amount to be refunded:	$
			charged:	$2182.00

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 10-0750/J&J1673/JWH in the amount of $2182.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 10-0750/J&J1673/JWH. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Docket No. J&J-1673

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Kutchan, Toni M., Zenk, Meinhart H., Atkins, David G., Frost, Anthony J.

Serial No. : Art Unit:

Filed : February 28, 2000 Examiner:

For : **"CYTOCHROME P450 REDUCTASES FROM POPPY PLANTS"**

Express Mail #EL327261263US
Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Please amend the above identified application as follows:

IN THE CLAIMS

In claim 3, line 7, , delete "1 or claim".

In claim 4, line 12, delete "any one of the preceding claims" and insert -claim 3-- in its place.

In claim 9, line 25, delete "any one of claims 2 to 8" and insert --claim 3-- in its place.

In claim 12, lines 5 and 6, delete "any one of claims 1 to 11" and insert --claim 10-- in its place.

In claim 13, lines 7 and 8, delete "any one of claims 1 to 12" and insert --claim 1-- in its place.

In claim 15, line 10, delete "or claim 14".

In claim 16, line 13, delete "any one of claims 13 to 15" and insert --claim 13-- in its place.

In claim 19, lines 19 and 20, delete "any one of claims 1 to 11 or a DNA construct of any one of claims 13 to 18" and insert -claim 1-- in its place.

In claim 28, line 32, delete "any one of claims 19 to 27" and insert - claim 19-- in its place.

In claim 29, lines 2 and 3, delete "any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18" and insert --claim 1-- in its place.

In claim 37, lines 13 and 14, delete "any one of claims 1 to 11 or a DNZ construct according to any one of claims 13 to 18" and insert --claim 13-- in its place.

In claim 38, lines 15 and 16, delete "any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18" and insert - claim 13—in its place.

In claim 41, line 22, delete "claims 38 to 40" and insert --claim 38—in its place.

In claim 43, line 26, delete "or claim 42".

In claim 44, line 28, delete "or claim 42".

In claim 45, lines 32 and 33, delete"any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18" and insert --claim 1-- in its place.

In claim 52, lines 20 and 21, delete "any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18" and insert --claim 1-- in its place.

In claim 53, lines 24 and 25, delete "any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18" and insert --claim 1-- in its place.

In claim 54, lines 27 and 28, delete "or claim 53".

In claim 55, line 29, delete "any one of claims 52 to 54" and insert --claim 52—in its place.

In claim 57, lines 4 and 5, delete "any one of claims 52 to 55 or the concentrate according to claim 56" and insert --claim 52.--in its place.

In claim 59 a), lines 10 and 11, delete "any one of claims 1 to 12 or a DNA construct according to any one of claims 13 to 18" and insert --claim 1-- in its place.

In claim 60 a), lines 17 and 18, delete "any one of claims 1 to 12 or a DNA construct according to any one of claims 13 to 18" and insert --claim 1-- in its place.

In claim 61, line 23, delete "or claim 60".

Add the following:

--Claim 62. A method according to claim 60, whereas the alkaloid is selected from the group consisting of morphine, codeine, oripavine and thebaine.--

Respectfully Submitted

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"CYTOCHROME P450 REDUCTASES FROM POPPY PLANTS"

TECHNICAL FIELD

The present invention relates to production of alkaloids from poppy plants and in particular to genes encoding enzymes in the alkaloid pathway, to proteins encoded by the genes, to plants transformed or transfected with the genes and to methods of altering alkaloid content or blend of poppy plants.

INTRODUCTION

The opium poppy *Papaver somniferum* is grown under strict government control, for the production of medically useful alkaloids such as morphine and codeine. The alkaloid content of poppy straw (includes threshed poppy capsules) is the most important parameter in the efficiency of opium alkaloid production. There have been numerous attempts to increase the yield of alkaloid per ton of poppy material. The vast majority of approaches focus on improving agricultural practices and on established methods of conventional breeding in the attempt to increase cultivation efficiencies and to modifying the genotype of the opium poppy plants.

In addition to increasing the overall yield of opium alkaloids, the relative content of particular alkaloids in the poppy plants is also of considerable importance and has an impact on efficiency of processing of the plant material and the ultimate yield and cost of an alkaloid.

Usually only one of the many alkaloids that can be produced by a poppy plant is found as the predominant alkaloid. In the opium poppy this is predominantly morphine which accumulates after flowering of the plant. However, before flowering thebaine is most abundant. The reason for such bias can be explained, at least in part, by analysis of what is currently known about alkaloid metabolism in the opium poppy and its regulation.

The network of reactions, enzymes, co-factors and metabolic intermediates leading to the synthesis of alkaloids in the opium poppy constitute a complex metabolic pathway which is regulated at numerous points. There are also thought to be a number of rate limiting steps ("bottlenecks") where limitations in the availability of either substrates, co-factors or certain enzymes, determine which particular branch of the synthetic pathway is favoured and therefore the ultimate "mix" of alkaloids and the type of alkaloid which is predominantly in the plant. A class of enzymes known as cytochrome P-450 are known to be involved in the synthesis of several intermediates in the pathway. However, unlike the enzymology of mammalian cytochrome P450

enzymes, similar plant enzymes are considerably less abundant (Biochimie 1987, **69**:743-752) and have been less clearly described. It is known that plant P450 enzymes are like mammalian proteins and that they are hemoproteins which have a common prosthetic group containing iron and are membrane-bound proteins found within the endoplasmic reticulum. Generally, the P450-dependent enzymes catalyse the transferral of oxygen to the substrate and effectively remove one of the atoms from an oxygen molecule and are also referred to as monooxygenases. The reactions are dependent on a range of co-factors including NADPH and a second enzyme P450 cytochrome reductase.

More particularly, in the biosynthesis of alkaloids in plants, cytochrome P-450-dependent oxidases and monooxygenases have been shown to catalyse highly regio- and stereoselective reactions. Hydroxylases and oxidases specific to alkaloid biosynthesis have been identified and characterised for the protopine, berberine, bisbenzylisoquinoline, benzophenanthridine, morphinan and monoterpenoid indole alkaloid biosynthetic pathways. The role of cytochrome P-450s in alkaloid biosynthesis is exemplified by the biosynthesis of sanguinarine in *Eschscholzia californica* (California poppy). Of the six oxidative transformations involved in the conversion of (*S*)-reticuline to sanguinarine, four are thought to be catalysed by cytochrome P-450-dependent enzymes.

Thus, plant cytochrome P-450-dependent enzymes, including those from the alkaloid poppy, constitute a substrate-specific class of enzymes that differs from their mammalian counterpart in the high regio- and stereospecificity as well as in the novel nature of the reactions catalysed. The enzymes involved in the alkaloid biosynthetic pathway require among other things the presence of a cytochrome P-450 reductase enzyme. Plant cytochrome P-450 reductases have been purified or enriched from *C. roseus* (1, 8) sweet potato (9), *Helianthus tuberosus* (Jerusalem artichoke) (10), *Glycine max* (soybean) cell suspension cultures (11), *Pueraria lobata* (12) and petunia flowers (13). cDNA encoding cytochrome P-450 reductase has been isolated from *Vigna radiata* (mung bean) (14), *C. roseus* (15), *H. tuberosous* (accession Z26250, Z26251), *Vicia sativa* (accession Z26252) and *Arabidopsis* (16). cDNA cloning and heterologous expression in *E. coli* of the *C. roseus* cytochrome P-450 reductase has been reported (15).

The reductase is responsible for providing electrons to the P450 and is thought to be a relatively promiscuous enzyme in that a particular reductase species will reduce a range of distinct P450s. It is also known that the cytochrome P450 enzymes are in molar

excess to the level of P450 reductase. This imbalance may be a regulatory step for the reduction and therefore be rate-limiting of the cytochrome P450 activity. Although there is some promiscuity within species, available data suggests that there is poor transferability of reductases from diverged species. For example, although cytochrome P-450 reductase from insect cell culture and porcine liver was shown to transfer electrons to heterologously expressed *Berberis* berbamunine synthase, the highest turnover number was achieved with the *Berberis* reductase (7).

Notwithstanding this body of work, to date it has not been possible to establish the exact nature of the "bottlenecks" in the alkaloid metabolism pathway or to identify the key enzymes which may be responsible and which could be used to manipulate alkaloid metabolism in the opium poppy in order to achieve higher yields of alkaloids generally, and specific alkaloids in particular.

As the cost of producing poppy alkaloids is very dependent on the alkaloid content of poppy straw, it would be a major advantage if high alkaloid containing straw could be obtained rather than to attempt to increase the yield of straw. In fact, it is possible that any increase in the yield of straw may result in the relative content of alkaloid decreasing through dilution. High alkaloid-containing straw would provide efficiencies throughout the CPS ("Concentrate of Poppy Straw") production process. If high crop yields can be achieved, either less hectares of crop need be grown or the pre-existing areas can be used to increase production. High yield crops would also reduce the cost of harvest, transport, drying, storage, processing and waste disposal per unit weight of alkaloid. Thus, to increase the yield of an alkaloid it would be most efficient to manipulate the plants to increase alkaloid content of the straw rather than to increase the yield of straw.

SUMMARY OF THE INVENTION

It has now been found that among the rate-limiting steps in the production of morphine in *Papaver somniferum* are the steps which depend on the cytochrome P-450 enzymes, and therefore in turn on the cytochrome P-450 reductases. This observation has led to the identification and isolation of cytochrome P-450 reductase enzymes in the alkaloid poppy, the isolation and characterisation of polynucleotides encoding the reductase enzymes, the expression of the polynucleotides encoding the reductases in eukaryotic and prokaryotic expression systems, including plant cells and transfected or transformed plants. The identification of cytochrome P-450 reductase genes and their products in poppy plants now enables methods of controlling the total alkaloid content

of a plant, the ultimate "mix" of alkaloids as well as the type of predominant alkaloid synthesised by the plant. This can be achieved by alleviating the "bottlenecks" in the pathway through overexpression of the relevant reductase genes in plants transformed or transfected with a nucleotide sequence encoding an appropriate P-450 reductase enzyme.

Thus, according to a first aspect there is an isolated and purified polynucleotide encoding a cytochrome P-450 reductase enzyme from an alkaloid poppy plant, or a variant, fragment or analog thereof.

The polynucleotide may be selected from the group consisting of genomic DNA (gDNA), cDNA, or synthetic DNA. The preferred polynucleotides encoding a cytochrome P-450 reductase are selected from those shown in Figures 9a and 9b or fragments thereof. It will be understood however that sequences shown in the Figures 9a and 9b may be expressed in the absence of the native leader sequences or any of the 5' or 3' untranslated regions of the polynucleotide. Such regions of the polynucleotide may be replaced with exogenous control/regulatory sequences in order to optimise/enhance expression of the sequence in an expression system. Figures 10a and 10b represent examples of truncated polynucleotide sequences encoding cytochrome P-450 reductases of *P. somniferum* and *E. californica* respectively, wherein the native leader sequences have been removed in order to enhance the expression of the enzyme.

The preferred alkaloid-producing poppy plants are *Eschscholzia californica* and *Papaver somniferum.*

It will also be understood that analogues and variants of the polynucleotide encoding a cytochrome P-450 reductase from alkaloid poppy plants fall within the scope of the present invention. Such variants will still encode an enzyme with cytochrome P-450 reductase properties and may include codon substitutions or modifications which do not alter the amino acid encoded by the codon but which enable efficient expression of the polynucleotide encoding cytochrome P-450 reductase enzyme in a chose expression system. Other variants may be naturally occurring, for example allelic variants or isoforms.

According to a second aspect there is provided an isolated and purified polynucleotide having a sequence which is complementary to all or part of the sequence of a polynucleotide according to the first aspect.

Such complementary polynucleotides are useful in the present invention as probes and primers, as antisense agents or may be used in the design of other suppressive agents such as ribozymes and the like.

According to a third aspect there is provided an isolated and purified polynucleotide which codes for prokaryotic or eukaryotic expression of a cytochrome P-450 reductase enzyme from an alkaloid poppy plant, or a variant, analog or fragment thereof, wherein the polynucleotide is expressed in an environment selected from the group consisting of the extracellular environment, an intracellular membranous compartment, intracellular cytoplasmic compartment or combinations thereof.

The polynucleotide encoding a cytochrome P-450 reductase may be coupled to another nucleotide sequence which would assist or directing the expression of the reductase with respect to a particular cellular compartment or the extracellular environment.

According to a fourth aspect there is provided a recombinant DNA construct comprising the polynucleotide according to any one of first to third aspects.

Preferably the recombinant DNA construct is a viral or plasmid vector. Such a vector may direct prokaryotic or eukaryotic expression of the polynucleotide encoding a cytochrome P-450 reductase or it may prevent or reduce its expression.

According to a fifth aspect there is provided an isolated and purified cytochrome P-450 reductase enzyme, being a product of prokaryotic or eukaryotic expression of the polynucleotide of any one of first to third aspects or a DNA construct of the fourth aspect.

Variants of the cytochrome P-450 reductase enzyme which incorporate amino acid deletions, substitutions, additions or combinations thereof, are also contemplated. The variants can be advantageously prepared by introducing appropriate codon mutations, deletions, insertions or combinations thereof, into the polynucleotide encoding the P-450 reductase enzyme. Such variants will retain the properties of the P-450 reductase enzyme, either *in vivo* or *in vitro*. Other variants may be naturally occurring, for example allelic variants or isoforms.

The cytochrome P-450 reductase may be expressed in and by a variety of eukaryotic and prokaryotic cells and organisms., including bacteria, yeasts, insect cells, mammalian and other vertebrate cells, or plant cells. Preferably the expression system is a plant expression system and even more preferred is an alkaloid poppy plant. Suitable alkaloid poppy plants are *Eschscholzia californica* and *Papaver somniferum*.

For expression of cytochrome P-450 reductase activity, a fragment of the polynucleotide encoding a cytochrome P-450 reductase may be employed, such fragment encodes functionally relevant regions, motifs or domains of the reductase

protein. Similarly, fragments of the P-450 reductase enzyme resulting from the recombinant expression of the polynucleotide may be used. Functionally important domains of cytochrome P-450 reductase may be represented by individual exons or may be identified as being highly conserved regions of the protein molecule. Those parts of the cytochrome P-450 reductase which are not highly conserved may have important functional properties in a particular expression system.

According to a sixth aspect there is provided a cell transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect.

Cells which may be transfected or transformed with a polynucleotide encoding a cytochrome P-450 reductase are bacterial, yeast, animal or plant cells. For preference the cells are plant cells. Even more preferred are cells from an alkaloid poppy plant, such as *Eschscholzia californica* or *Papaver somniferum.*

According to a seventh aspect there is provided a method for preparing plants which overexpress a cytochrome P-450 reductase enzyme, comprising transfecting or transforming a plant cell, a plant part or a plant, with the polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect.

Preferably, the plants overexpressing the P-450 reductase are *Eschscholzia californica* and *Papaver somniferum.* Suitable promoters to control the expression of the P-450 reductase gene may be derived from for example cauliflower mosaic virus or subterranean clover mosaic virus. Other virus promoters may also be suitable. Further, the use of the endogenous promoter may also be appropriate in certain circumstances. Such a promoter may be co-isolated with the gDNA encoding the P-450 reductase enzyme.

According to a eighth aspect there is provided a plant transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, wherein the plant exhibits altered expression of the cytochrome P-450 reductase enzyme

For preference, the altered expression manifests itself in overexpression of the cytochrome P-450 reductase enzyme. However, reduced expression of cytochrome P-450 reductase can also be achieved if the plant is transformed or transfected with a polynucleotide which is complementary to the polynucleotide encoding the reductase.

Even more preferably, the transformed or transfected plant is an alkaloid poppy plant, wherein the plant has a higher or different alkaloid content when compared to a plant which has not been so transformed or transfected.

Preferably the transformed or transfected plants having higher or different alkaloid content are selected from *Eschscholzia californica* and *Papaver somniferum.* Even more preferred is *Papaver somniferum.*

According to a ninth aspect there is provided a method of altering the yield or type of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, or with a polynucleotide which binds under stringent conditions to the polynucleotide encoding said enzyme.

According to a tenth aspect there is provided a method of increasing the yield of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, wherein the enzyme is overexpressed in said plant

According to a eleventh aspect there is provided a method of altering type or blend of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, or with a polynucleotide which binds under stringent conditions to the polynucleotide encoding said enzyme.

According to a twelfth aspect there is provided a stand of stably reproducing alkaloid poppies transformed or transfected with a polynucleotide according to any one first to third aspects or a DNA construct according to the fourth aspect, having altered expression of the cytochrome P-450 reductase enzyme.

According to a thirteenth aspect there is provided a stand of stably reproducing alkaloid poppies transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, having a higher or different alkaloid content when compared to a plant which has not been so transformed or transfected.

Preferably the stably reproducing alkaloid poppy is *Papaver somniferum.*

According to a fourteenth aspect there is provided straw of stably reproducing poppies according twelfth or thirteenth aspect, having a higher or different alkaloid content when compared to the straw obtained from an alkaloid poppy which has not been transformed or transfected.

According to a fifteenth aspect there is provided a concentrate of straw according to the fourteenth aspect, having a higher or different alkaloid content when compared to the concentrate of straw obtained from an alkaloid poppy which has not been transformed or transfected.

According to a sixteenth aspect there is provided an alkaloid when isolated from the straw according to fourteenth aspect or the concentrate according to the fifteenth aspect.

According to a seventeenth aspect there is provided a method for the production of poppy plant alkaloids, comprising the steps of;

a) harvesting capsules of an alkaloid poppy plant transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, to produce a straw where the poppy plant is such a plant that the straw has a higher or different alkaloid content when compared to the straw obtained from a poppy plant which has not been transformed or transfected.

b) chemically extracting the alkaloids from the straw.

According to an eighteenth aspect there is provided a method for the production of poppy alkaloids, comprising the steps of;

a) collecting and drying the latex of the immature capsules of an alkaloid poppy plant transformed or transfected with a polynucleotide according to any one of first to third aspects or a DNA construct according to the fourth aspect, to produce opium where the poppy plant is such a plant that the opium has a higher or different alkaloid content when compared to the opium obtained from a poppy plant which has not been transformed or transfected.

b) chemically extracting the alkaloids from the opium.

For preference the alkaloid is morphine, codeine, oripavine or thebaine, but it will be understood that other intermediates in the alkaloid metabolic pathway are also within the scope of the present invention, as are mixtures of alkaloids.

Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

BRIEF DESCRIPTION OF FIGURES

Figure. 1. SDS-PAGE analysis of fractions from the purification of cytochrome P-450 reductase from *P. somniferum* cell suspension cultures. Protein bands were visualised by silver staining. Lane 1, protein standards; lane 2, affinity chromatography elution buffer without protein: lane 3, 1 μg protein from the 2',5'-ADP Sepharose 4B eluate after dialysis; lane 4, 4 μg microsomal protein; lanes 5,6, 4 μg solubilized microsomal protein; lane 7, 4 μg protein from the DEAE cellulose eluate.

Figure 2. Amino acid sequences of seven endoproteinase Lys-C-generated peptides of the cytochrome P-450 reductase from *P. somniferum* cell suspension cultures.

Figure 3. Partial amino acid sequence comparison of plant cytochrome P-450 reductases. The shaded areas and arrows indicate the position and direction of the regions used for PCR oligodeoxynucleotide primer design.

Figure 4. Genomic DNA gel blot analysis of (A) *P. somniferum* hybridized to the *P. somniferum* full-length cDNA and (B) *E. californica* hybridzed to the *E. californica* full-length cDNA and to (C) the 288 bp PCR fragment corresponding to the second isoform. The numbers following the restriction enzyme names indicate the number of recognition sites that occur in the reading frame. For the second *E.californica* isoform, this is known only over a 288 bp region.

Figure 5. Comparison of the amino acid sequences of the cytochrome P-450 reductase from *P. somniferum* and from *E. californica*. Top sequence, *E. californica*; bottom sequence, *P. somniferum*; *, amino acid identity.

Figure. 6. Nucleotide sequences of cDNA from (a) *P. somniferum*, and (b) *E. californica*.

Figure 7. Functional expression of cytochrome P-450 reductases in yeast and insect cell culture. (A) Expression of pYES2/PsoCPRI (———), pRS405/PsoCPRII (-·-·-·-·-), pYES2/PsoCP (·····), control (·····); (B) pFastBac/PsoCPRII (———), control (·····); (C) pYES2/EcaCPRII (———), pRS405/EcaCPRII (-·-·-·-·-), control (·····); (D) pFastBac/EcaCPRII (———), control (·····). Pso CPR, *P. somniferum* cytochrome P-450 reductase; Eca CPR, *E. californica* cytochrome P-450 reductase; Sf9, *S. frugiperda* Sf9 cell culture.

Figure 8. Restriction enzyme map (unique sites) for cDNA sequences of (a) *P. somniferum*, and (b) *E. californica*.

Figure 9. Amino acid sequences of (a) *P. somniferum*, and (b) *E. californica*, predicted from their respective cDNA nucleotide sequences. The start and stop codons are depicted in bold.

Figure 10. cDNA nucleotide sequences and their predicted amino acid sequences, of fragments subcloned into an expression vector: (a) *P. somniferum*, and (b) *E. californica*. Both sequences are truncated versions of sequences represented in Figures 9a and 9b, lacking the leader sequences. Extra vector sequences/restriction sites derived during subcloning are shown in lowercase and the cDNA in uppercase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The details of the metabolic pathway leading to synthesis of opium alkaloids in the opium poppy, *Papaver somniferum,* part of which is depicted in Scheme I. Typically, the P450 enzyme exists in a 15 - 20 fold excess as compared to the reductase level and as there is approximately a 6:1 dependence between the two enzymes, it is feasible that the reductase levels are limiting the rate of the cytochrome P450 enzyme. By supplying plant tissue with radiolabeled compounds and following the accumulation of radioactivity in the various intermediates in the pathway it was shown that addition of radiolabel (labelled reticuline, salutaridinol) before thebaine results in accumulation of radioactivity at thebaine. Addition of radiolabeled compounds after thebaine result in the accumulation of radioactivity at codeine.

Oripavine is an intermediate from a second route of conversion from thebaine to morphine. It is thought that thebaine is converted to oripivine by the same 3 demethylase that converts codeine to morphinone. The slow modification of the isotopic oripavine is probably due to the rate limitation of the 6 demethylase.

With the assistance of such experiments it has now been found that among the rate-limiting steps in the production of morphine in *Papaver somniferum* are the steps which depend on the reduction of cytochrome P-450 by the cytochrome P-450 reductases.

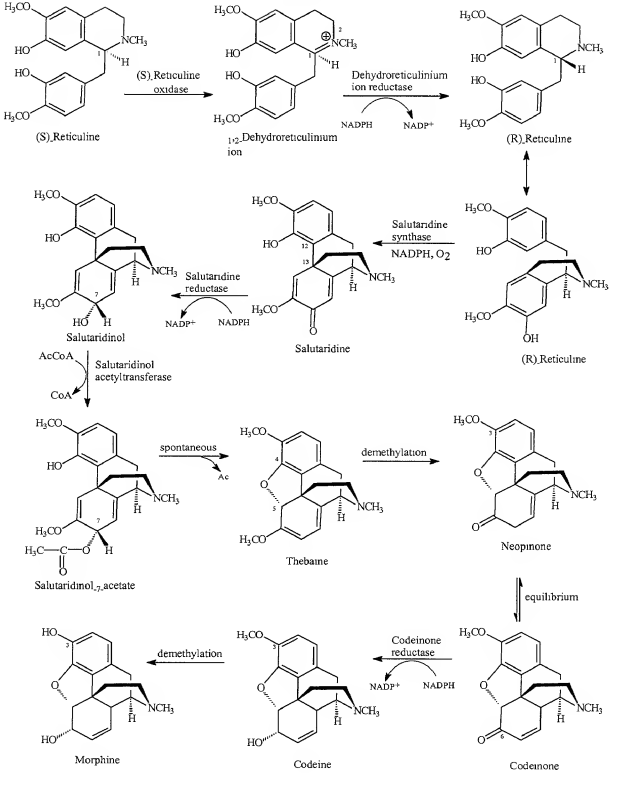
Thus the following steps are known or suspected to be catalysed by P-450 enzymes which are rate limiting:

1 (R)-reticuline → salutaridine

2 thebaine → neopinone → codeinone

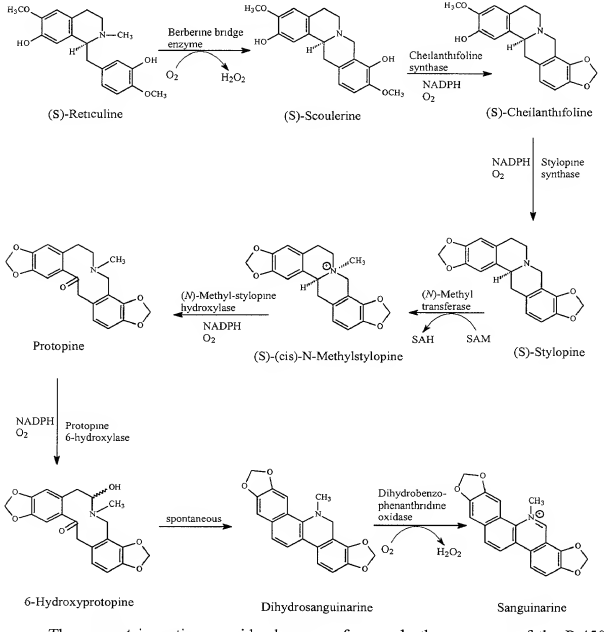
3 codeine → morphine

SCHEME I


(S)-Reticuline
(S)-Reticuline oxidase
1,2-Dehydroreticulinium ion
Dehydroreticulinium ion reductase
NADPH
NADP+
(R)-Reticuline
Salutaridine synthase
NADPH, O2
Salutaridine
Salutaridine reductase
NADP+
NADPH
Salutaridinol
AcCoA
CoA
Salutaridinol acetyltransferase
Salutaridinol-7-acetate
spontaneous
Ac
Thebaine
demethylation
Neopinone
equilibrium
Codeinone
Codeinone reductase
NADP+
NADPH
Codeine
demethylation
Morphine

The importance of cytochrome P-450 in alkaloid biosynthesis is also exemplified by the biosynthesis of sanguinarine in *Eschscholzia californica* (California poppy). This biosynthetic pathway is shown in Scheme II.

SCHEME II



The present invention provides by way of example the sequence of the P-450 reductase enzyme genes from two poppy species, the opium poppy *Papaver somniferum* and the Californian poppy *Eschscholzia californica*. The sequence information has been shown to code for the enzymes by expression in a heterologous expression system followed by biochemical characterisation. These studies have also shown that the

alteration of the ratio and species of reductase will significantly alter the interaction of the cytochrome P-450 with its substrate suggesting a lack of tolerance for general interchange of reductase genes.

The over-expression of the P-450 reductase gene in an alkaloid producing plant will alleviate the rate-limitation of the P450 by increasing the rate of reduction of the active P-450 enzyme. In brief, controlling the reductase should control the P-450 cytochrome.

The information on the protein coding region of cytochrome P-450 reductase enzymes may be applied to increase yields of alkaloids in the poppy plant as follows:

1) obtain the gDNA or cDNA sequence of the gene from the target plant and a closely related plant.

2) sub-clone the gDNA or cDNA into a plasmid vector that contains the following:

- a promoter suitable for overexpression of the cDNA in poppy, for example a promoter derived from the cauliflower mosaic virus or the subterranean clover mosaic virus.
- a selectable marker linked to a different promoter to facilitate the selection of transformants. Marker could be a dominant marker such as a herbicide resistance gene or an antibiotic resistance gene.
- suitable selectable markers and replication origins for maintenance of the plasmid in bacteria
- suitable sequences to facilitate mobilisation of the plasmid by *Agrobacterium tumefacians*-mediated transformation.

3) transform a suitable strain of *A. tumefacians* and then co-cultivate the bacteria with suitable samples of plant tissue such as callus, embryonic tissue or hypocotyl tissue.

4) place treated tissue on selectable media and provide appropriate media to promote differentiation and plant re-generation.

5) characterise candidate plants by Southern and Northern blotting to confirm integration of gene and expression in appropriate tissues

6) self-pollinate transformed plants, analyse segregants to identify hemizygotes and homozygotes

7) analyse biochemistry of transgenic plants.

Isotope labelling can be used to identify bottlenecks and HPLC analysis will determine levels of alkaloids.

In order to develop optimised convenient heterologous expression systems for the cytochrome P-450-dependent oxidases of select isoquinoline alkaloid-producing plant species, facile PCR-based method have been developed with which to clone cytochrome P-450 reductase and express the enzyme in yeast and insect cell culture as initial expression systems.

The invention will now be described with reference to specific examples.

EXAMPLES

Example 1: Enzyme purification and amino acid sequencing:

Plant cell cultures. Cell suspension cultures of *P. somniferum* and *E. californica* were routinely grown in 1-litre conical flasks containing 400 mL of Linsmaier-Skoog medium (17) over 7 days at 23°C on a gyratory shaker (100 rpm) in diffuse light (750 lux). Elicitation of *E. californica* cell suspension cultures was achieved by the aseptic addition of methyl jasmonate to a final concentration of 100 μM to the medium (18).

Purification and sequence analysis. Cells were harvested from seven-day-old suspension cultures of *P. somniferum* by vacuum filtration, immediately shock frozen and stored at -20°C. All of the following operations were carried out at 4°C. 500 g frozen tissue were then homogenised with a mortar and pestle in 1 litre 0.1 M tricine/NaOH, pH 7.5 containing 15 mM thioglycolic acid. Cell debris was removed by centrifugation at 10,000 x g, 30 min. The supernatant was filtered through four layers of cheesecloth and the microsomes were then isolated by $MgCl_2$ precipitation according to (19). In a typical preparation, 500 g fresh weight of cells yielded 8-10 mg/mL microsomal protein. Microsomal protein was solubilized as follows. 2 mg CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane-sulfonate, Roth) per mg microsomal protein was prepared in 1 mL of 0.1 M tricine/NaOH, pH 7.5 containing 15 mM thioglycolic acid. This solution was added dropwise to the microsomal suspension. 2% (v/v) Emulgen 911 (Kao Corporation) was then added and the solution slowly stirred for 1 h. Membrane fragments were removed by centrifugation at 105,000 x g for 60 min. The total activity in the solubilized microsomes was assigned the value 100%. The solubilized cytochrome P-450 reductase was then purified to electrophoretic homogeneity according to (20). In this manner, 50 μg cytochrome P-450 reductase was purified from 8 kg *P. somniferum* cell suspension culture in 97% yield.

The purified enzyme preparation was subjected to SDS/PAGE to remove traces of Emulgen 911 and CHAPS, and the Coomassie brilliant blue R-250-visualized band

representing the cytochrome P-450 reductase was digested *in situ* with endoproteinase Lys- C as reported in (21). The peptide mixture thereby obtained was resolved by reversed phase HPLC [column, Merck Lichrospher RP18; 5 μm (4 x 125 mm); solvent system, (A) 0.1% trifluoroacetic acid, (B) 0.1% trifluoroacetic acid / 60% acetonitrile; gradient of 1% per min; flow rate of 1mL/min] with detection at 206 nm. The scheme for the purification of the cytochrome P-450 reductase is given in Table I.

TABLE I Purification of Cytochrome P-450 Reductase from *P. somniferum* Cell Suspension Cultures

Purification Step	Total protein (mg)	Total activity (nkatal)	Specific activity (nkatal/mg)	Purification factor (fold)	Yield (%)
Microsomes	308	59	0.2	1	-
Solubilized microsomes	244	71	0.3	1	100
DEAE Cellulose	21	71	3.4	11	100
2',5'-ADP Sepharose 4B	0.05	47	927	3100	66
Dialysis	0.05	69	1385	4600	97

Following this facile purification procedure (20), 50 μg of enzyme could be purified to near electrophoretic homogeneity from 8 kg fresh weight of cell suspension culture with minimal loss of activity. Gel electrophoretic analysis of aliquots of the purification steps suggest that there may be two isoforms of the cytochrome P-450 reductase in *P. somniferum* as there were two protein bands present in the 2',5'-ADP Sepharose 4B eluate at 80 kDa (Fig. 1). To further test the possible presence of isoforms, 10 μg protein from the 2',5'-ADP Sepharose 4B eluate was subjected to native polyacrylamide gel electrophoresis, the two closely migrating protein bands were eluted and both tested positive for cytochrome *c* reduction. These two isozymes could not be chromatographically resolved and were therefore characterised together.

The purified reductase exhibited a pH optimum at 8.0 in 0.5 M Tricine buffer. The optimal molarity range of the Tricine buffer was determined to be 250-500 mM. At 100 mM and at 1 M Tricine, the activity declined to 21% and 77%, respectively. The K_m value for cytochrome *c* was 8.3 μM and that for the cofactor NADPH was 4.2 μM. The distribution of the cytochrome P-450 reductase in a 3-month-old *P. somniferum* plant is given in Table II.

TABLE II Distribution of Cytochrome P-450 Reductase Activity in a 3-Month-Old *P. somniferum* Plant

Plant part	Specific activity (pkatal/g dry weight)	Specific activity (pkatal/mg protein)
Capsule	2700	660
stem	2000	930
Leaf	840	390
Root	670	740

On a dry weight basis, the highest activity is present in the capsule.

Microsequencing was accomplished with an Applied Biosystems model 470 gas-phase sequencer. The amino acid sequence of seven endoproteinase Lys-C-generated peptides was determined on the mixture of both isozymes (Fig. 2). A comparison of these amino acid sequences with those available for plant cytochrome P-450 reductases in the GenBank/EMBL sequence database allowed the relative positioning of the seven internal peptides due to high sequence homology. This also served as supportive evidence that the isozymes that were purified were indeed cytochrome P-450 reductases.

Example 2: Generation of partial cDNAs from *P. somniferum* and *E. californica*.

Optimised PCR primers were then designed based on highly homologous sites on both the amino acid and nucleotide levels in the plant cytochrome P-450 reductase sequence comparison (Fig. 3). The precise sequence of the primers used for the first round of PCR was:

```
5'-CA ITI CII CCT CCT TTC CC-3'   and
   T

3'-ACC TAC TTC TTA CGI CAA GG-5'.
                C       TGC
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Polymerase chain reaction (PCR) generated partial cDNAs encoding cytochrome P-450 reductases from *P. somniferum* and *E. californica* were produced by PCR using cDNA produced by reverse transcription of total RNA isolated from 3 to 5-day-old suspension cultured cells. DNA amplification was performed under the following conditions: 5 cycles of 94°C, 30 sec; 45°C, 1 min; 72°C, 1 min; 25 cycles of 94°C, 30 sec; 55°C, 30 sec, 72°C, 1 min. At the end of 30 cycles, the reaction mixtures were incubated for an additional 5 min at 72°C prior to cooling to 4°C. The amplified DNA

was then resolved by agarose gel electrophoresis, the bands of approximately the correct size were isolated and subcloned into pGEM-T (Promega) prior to nucleotide sequence determination.

Resolution of this first PCR experiment by agarose gel electrophoresis revealed a mixture of DNA products in the expected range of 400-450 bp. The bands in this size range were eluted from the gel and used as template for nested PCR with the following primers:

```
5'-CA ITI CII CCT CCT TTC CC-3'  and
   T

3'-AAA CGI CGI TAI CGI GGI GCI IGI GTT GG-5'
      G                                  C
```

The result from the nested PCR was a single DNA band with the expected size of 288 bp. The translation of the nucleotide sequence of this PCR product indicated that it was indeed encoding a cytochrome P-450 reductase. This 288 bp PCR-generated partial cDNA was then used as hybridisation probe to screen an amplified *P. somniferum* cell suspension culture cDNA library. In this manner, from a total of 300,000 clones screened, two positive clones were isolated. Of these two positive clones, one was determine to be full-length by a restriction endonuclease analysis. The nucleotide sequence of this full-length cDNA clone was then determined for both strands. The reading frame coded for 684 amino acids corresponding to a relative molecular mass of 77.5 kDa.

An identical PCR-based approached was also carried out with RNA isolated from methyl jasmonate-induced *E. californica* cell suspension cultures (26). Nucleotide sequence determination of the 288 bp DNA fragment indicated that in *E. californica* one cytochrome P-450 reductase form is present. However, screening of 400,000 clones of a primary cDNA library prepared from RNA isolated from methyl jasmonate-induced *E. californica* cell suspension cultures resulted in the isolation of one partial and one full-length clone, both of which encoded a second isoform. The nucleotide sequence of this full-length cDNA clone was then determined for both strands. The reading frame encoded 705 amino acids that corresponded to a relative molecular mass of 78.7 kDa. RNA gel blot analysis indicated that this isoform gene is weakly induced two-fold by treatment of the cell cultures with methyl jasmonate. Genomic DNA gel blot analysis of

each reductase indicates that one gene encodes each isoform in *E. californica* and that one gene also encodes the cloned isoform in *P. somniferum* (Fig. 4).

The overall sequence homology of the cytochrome P-450 reductase from *P. somniferum* and that from *E. californica* is 63% identity at the nucleotide level and 69% identity at the amino acid level (Fig. 5). This compares to an overall sequence identity to other plant cytochrome P-450 reductases of approximately 50% at both the nucleotide and amino acid levels.

Nucleotide sequence determination. The entire nucleotide sequence on both DNA strands of full-length cDNA clones in pBluescript was determined by dideoxy cycle sequencing using internal DNA sequences for the design of deoxyoligonucleotides as sequencing primers. Nucleotide sequences of cDNAs of *P. somniferum* and *E. californica* are given in Figures 6A and 6B, respectively.

Alternative approaches. cDNA can also be prepared by isolating RNA from either plant cell suspension cultures or from different material, according to a method using LiCl precipitation of ribonucleic acid as described in "Current Protocols in Molecular Biology" Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds John Wiley & Sons, Inc. New York (1987). mRNA was then isolated from the total RNA using either an oligo dT cellulose column or oligo dT beads (Oligotex beads, QIAGEN) according to the manufacturers instructions. The cDNA libraries were prepared from mRNA with cDNA and lambda ZAP kits from Stratagen (La Jolla, California, USA), according to the manufacturers instructions.

Example 3: cDNA isolation and heterologous expression of cytochrome P-450 reductase in *Saccharomyces cerevisiae*.

cDNA clones encoding the *Papaver* and *Eschscholzia* cytochrome P-450 reductases were isolated by screening of cDNA libraries prepared in either l-ZAP II or Uni-ZAP XR (Stratagene) using the partial clones generated by PCR as hybridization probe. The clones that yielded positive results through a third screening were converted to pBluescript KS (+) by excision. After determination of the nucleotide sequence on both strands, the full length reading frame, free of the 5'- and 3'-flanking sequences, was generated by PCR using either *Taq* DNA polymerase (Perkin Elmer) and was subcloned into pGEM-T (Promega) or *Pfu* DNA polymerase and was subcloned into pCR-Script SK (+) (Stratagene).

The *P. somniferum* cytochrome P-450 reductase cDNA in pGEM-T, designated pGEM-T/PsoCPR, was digested with the restriction endonucleases *Not* I and *Hin* dIII and the 2096 bp fragment was ligated into *Not* I/*Hin* dIII digested pYES2 (autonomously replicating yeast expression vector from Invitrogen) to produce the expression plasmid pYES2/PsoCPRI. This particular construction had 27 bp of a noncoding region upstream from the AUG start codon. This was reduced to 6 bp by digestion of pYES2/PsoCPRI with *Hin* dIII and *Cla* I. This 55 bp restriction fragment was then replaced by ligation with a synthetic DNA adaptor of a sequence that replaced the reading frame from the internal *Cla* I site through the start codon, which was immediately preceded by a *Hin* dIII recognition sequence. The resulting construct was termed pYES2/PsoCPRII.

The *E. californica* cytochrome P-450 reductase cDNA in pGEM-T, designated pGEM-T/EcaCPR, was digested with the restriction endonucleases *Sal* I and *Not* I and the 2289 bp fragment was ligated into *Sal* I/*Not* I digested pGEM-9Zf (-) (Promega). pGEM-9Zf/EcaCPR was then digested with *Sst* I and *Not* I and the 2292 bp fragment was ligated into *Sst* I/*Not* I digested pYES2 to produce the expression plasmid pYES2/EcaCPRI. The noncoding sequences upstream of the start codon were minimized by digestion with *Sma* I and *Eco* ICRI and the vector recircularized by blunt-end ligation (plasmid termed pYES2/EcaCPRII). These autonomously replicating expression plasmids were then introduced into the *Saccharomyces cerevisiae* strain INVSC1 under uracil selection.

Expression of the vector pYES2/PsoCPRI containing 27 noncoding nucleotides upstream of the start codon resulted in increased enzyme activity that was 2.6-fold greater than the yeast endogenous reductase (Fig. 7A). Shortening of this noncoding sequence to 6 bp in vector pYES2/PsoCPRII resulted in 9-fold greater enzyme activity than in the control yeast strain containing only the vector pYES2. Expression using the integrative yeast vector pRS405 was also investigated for the eventual possible heterologous co-expression of both a plant cytochrome P-450 reductase and a plant oxidase in yeast. Expression of the vector pRS405/PsoCPRII, in which transcription of the *P. somniferum* cytochrome P-450 reductase was also driven by the *GAL1* gene promoter, resulted in 67% of the enzyme activity compared to the autonomously replicating vector pYES2/PsoCPRII.

Expression of pYES2/EcaCPRII and of pRS405/EcaCPRII resulted in a 15-fold and 10-fold increase in activity over the endogenous yeast reductase, respectively (Fig. 7C).

The *P. somniferum* cytochrome P-450 reductase reading frame downstream from the GAL 1 promoter was generated by PCR from pYES2/PsoCPRII. The 2598 bp PCR product was ligated into pCRScript and then excised by digestion with *Not* I and *Sal* I. This 2669 bp *Not* I/*Sal* I fragment was ligated into the *Not* I/*Sal* I digested yeast integrative expression vector pRS405 (Stratagene).

The *E. californica* cytochrome P-450 reductase reading frame downstream from the GAL 1 promoter was introduced into the yeast integrative expression vector pRS405 by digestion of pYES2/EcaCPRII with *Pst* I and *Not* I and the 2835 bp fragment was ligated into *Pst* I/*Not* I digested vector. The integrative expression plasmids pRS405/PsoCPRII and pRS405/EcaCPRII were then introduced into the *S. cerevisiae* strain INVSC1 under leucine selection.

Yeast microsomes were isolated according to either (22) or (23) and the presence cytochrome P-450 reductase was measured as the ability to reduce cytochrome *c* (24).

Example 4: Heterologous expression of cytochrome P-450 reductase in *Spodoptera frugiperda* Sf9 cells.

The *P. somniferum* cytochrome P-450 reductase cDNA construct pYES2/PsoCPRII was digested with *Hin* dIII and *Xba* I and the resultant 2096 bp fragment was ligated into *Hin* dIII/*Xba* I digested pGEM-7Zf (+) (Promega). pGEM-7Zf/PsoCPRII was then digested with *Bam* HI and *Xho* I and the 2090 bp fragment was ligated into *Bam* HI/*Xho* I digested pFastBac1 (Life Technologies).

The *E. californica* cytochrome P-450 reductase clone pGEM-T/EcaCPRII was digested with the restriction endonucleases *Sma* I and *Not* I and the 2251 bp fragment was ligated into pFastBac1 that had been digested first with *Bam* HI, then with *Pfu* DNA polymerase to produce blunt ends, and finally with *Not* I. pFastBac/PsoCPRII and pFastBac/EcaCPRII were transposed into baculovirus DNA and then transfected into *Spodoptera frugiperda* Sf9 cells according to the manufacturer's instructions. The insect cells were propagated and the recombinant virus was amplified according to (7). Isolation of insect cell microsomes was performed as published (7) and the cytochrome *c* reducing activity measured as for the yeast expression.

Heterologous expression in insect cell culture (*S. frugiperda* Sf9 cells) of pFastBac/PsoCPRII produced 4-fold more activity than the insect cell endogenous reductase, representing 40% of the activity produced by pYES2/PsoCPRII in yeast (Fig. 7B). Expression of pFastBac/PsoCPRI, the construction containing a 27 bp long 5'-noncoding region, resulted in no measurable enzyme activity above that from the endogenous insect cell reductase.

Expression of pFastBac/EcaCPRII in insect cell culture produced a 10-fold increase in reductase activity (Fig 7D). The overexpression in insect cell culture was 54% of that achieved in yeast.

Example 5: Co-expression of cytochrome P-450 reductase and berbamunine synthase in Sf9 cells.

To test for the possible effects of a plant cytochrome P-450 reductase as opposed to either yeast or insect cell reductase, several coexpressions were undertaken.

Recombinant baculovirus containing either the *P. somniferum* or *E. californica* cytochrome P-450 cDNA was added simultaneously to *S. frugiperda* Sf9 cells (Gibco-BRL) with recombinant virus containing the berbamunine synthase (CYP 80) cDNA (7). The oxidase virus was infected at a multiplicity of infection (MOI) of approximately 5 and the amount of reductase virus varied from an MOI from1-5. The infection were carried out as described in (25).

The cytochrome P-450 oxidase that was used in these experiments was the *C-O* phenol coupling enzyme of bisbenzylisoquinoline alkaloid biosynthesis from *B. stolonifera*, berbamunine synthase (7). In the presence of equimolar concentrations of the two substrates (*S*)-*N*-methylcoclaurine and (*R*)-*N*-methylcoclaurine, the native enzyme produces two products in a ratio of 90:10 (berbamunine (*R,S*-dimer):guattegaumerine (*R,R*-dimer)) that correspond to the ratio of these two alkaloids found in the *Berberis* plant (6). Berbamunine synthase expressed in insect cells produced, however, the dimers *R,S*:*R,R* in a ratio of 15:85 (7). Co-infection of insect cell culture with two baculovirus preparations, one containing berbamunine synthase cDNA and the other containing *E. californica* cytochrome P-450 reductase, in varying ratios resulted in a shift in the ratio of the enzymatic products formed as follows: oxidase:reductase (5:1), *R,S*:*R,R* (29:71); oxidase:reductase (1:1), *R,S*:*R,R* (35:65); oxidase:reductase (1:2), *R,S*:*R,R* (37:63).

The isolation and functional expression of cDNAs encoding cytochrome P-450 reductases from *E. californica* and *P. somniferum* described above were undertaken to develop suitable heterologous expression systems optimal for the active expression of select cytochrome P-450-dependent oxidases of alkaloid biosynthesis, thus providing a convenient test system. Initial characterisation of the cytochrome P-450 reductase from *P. somniferum* indicated that with respect to molecular weight, K_m and pH optimum, the reductase is similar to those characterised from other plant species (9,13,14). The purified reductase resolved into two closely migrating bands on SDS-PAGE, suggesting that isoforms are present in *P. somniferum*. This is similar to the finding that multiple reductase isoforms are present in *Arabidopsis thaliana* (16) and *H. tuberosus*. The presence of isoforms in *P. somniferum* was further supported by amino acid sequence analysis of the purified reductase as compared to the sequence identified through cDNA cloning. In addition, isolation of a cDNA encoding cytochrome P-450 reductase from *E. californica* indicated the presence of two isoforms in this plant species as well. The presence of at least two genes in each genome was corroborated by genomic DNA gel blot analysis.

The cDNA encoding one cytochrome P-450 reductase isoform from each *P. somniferum* and *E. californica* was functionally expressed in yeast in an autonomously replicating vector and in an integrative vector with transcription under the control of the *GAL1* gene promoter. These vector constructions resulted in a 6- to 15-fold increase in reductase activity as compared to the activity from the endogenous yeast reductase alone. Likewise, expression of the reductases in insect cell culture using a baculovirus expression vector produced a 4- to 10-fold increase in reductase activity. Improved heterologous expression was obtained when the 5'- noncoding sequences were completely removed from the cDNAs.

Co-expression of the *Eschscholzia* reductase with the plant oxidase berbamunine synthase (7) in insect cell culture indicated that the amount of plant reductase present exerted an influence on the ratio of the products that were enzymatically formed. A first indication of this effect was shown by reconstitution of purified heterologously expressed berbamunine synthase reconstituted with *Berberis* reductase or with porcine reductase (7). Since it is difficult to standardise the lipids when reductase and oxidase are purified from microsomal membranes originating from different organisms, a co-infection of insect cells with reductase and oxidase is one method by which to avoid the varying effects of lipids. An increasing amount of plant reductase resulted in a shift in

the ratio of products formed by berbamunine synthase from *R,S:R,R* in a ratio of 15:85 in the absence of *Eschscholzia* reductase to *R,S:R,R* (37:63) when a two-fold excess of baculovirus containing the *Eschscholzia* reductase was used for the co-infection. These results indicate that the cytochrome P-450 reductase may influence the binding of substrate to berbamunine synthase. Although the FMN, FAD and NADPH-binding domains of cytochrome P-450 reductase have been identified by sequence comparisons with well studied flavoproteins, less is known about the substrate binding sites (27,28). Interaction with the non-physiological substrate cytochrome *c* has been demonstrated by chemical cross-linking (29) and by site-directed mutagenesis (30) to involve an acidic region between amino acid residues 200-220 of rat cytochrome P-450 reductase, but an elucidation of the specific interaction between reductase and cytochrome P-450 has not yet been reported.

Example 6: Transformation of poppy plant cells with nucleotide sequences encoding cytochrome P-450 reductase proteins.

Plant material

The genotypes of *Papaver somniferum* used was C 048-6-14-64 obtained from Tasmanian Alkaloids, Australia. Seeds were surface sterilised by washing for 30-60 seconds in 70% ethanol then in 1%(w/v) sodium hypochlorite solution plus 1-2 drops of autoclaved Tween 20 or Triton X for 20 minutes with agitation. Seeds were rinsed three to four times in sterile distilled water or until no smell of bleach remains and placed on 90 x 25 mm Petri dishes containing B5O medium (see below). Dishes were sealed with Micropore tape and were usually stored at 4°C for 24 to 48 hours. Seeds were germinated at 24°C in a 16 hour light-8 hour dark cycle. Hypocotyls were excised from seedlings after 7-8 days of culture and were cut into 3-6 mm explants (usually 1-3 explants per seedling) and used in transformation experiments.

Tissue culture media and conditions

All culture media consisted of B5 macronutrients, micronutrients, iron salts and vitamins (32) and 20g/L sucrose. pH was adjusted with 1M KOH to pH 5.6, media was buffered with 10mM MES (2-[N-Morpholino]ethanesulfonic acid) and the gelling agent was 0.8% Sigma Agar. Growth regulators were added to media prior to autoclaving at 121°C for 20 minutes. B5O has no growth regulators and Callusing Medium (CM) has 1mg/L 2,4-D. Antibiotics were added after autoclaving and cooling to 55-65°C. Explant and type I callus cultures were grown in Petri dishes sealed with Micropore tape at 24°C. Type II callus and somatic embryos were cultured at 18°C.

Bacterial strains and binary vectors

The disarmed *Agrobacterium tumefaciens* strains AGLO and AGL1 (33) were used in transformation experiments. DNA constructs were based on the binary vector pPZP201 (34), e.g. pTAB101, with 35S 5':pat:35S 3'. Agrobacterium strains were maintained in glycerol at -80°C or on LB agar plates plus appropriate selection at 4°C. Fresh cultures were grown overnight at 28°C in 10 mL MG broth (35) without antibiotics. This Agrobacterium suspension was diluted to approximately $5x10^8$ cells mL^{-1} (OD_{600} = 0.25) for use in transformation experiments.

Transformation and embryogenesis

Hypocotyls were excised from seedlings and immediately inoculated by immersion in liquid Agrobacterium culture for 10-15 minutes. Explants were then transferred directly to CM. After four to five days co-cultivation explants were washed in sterile distilled water, until the water was clear of Agrobacterium, blotted on sterile filter paper and transferred to CM containing 150 mg/L Timentin plus 10 mg/L PPT (phosphinothricin, the active ingredient of Basta herbicide). Explants were transferred to fresh CM at three weekly intervals. They initially produced friable brownish type I callus which subsequently formed small regions of very white, compact embryogenic callus (type II) by about 7-8 weeks culture.

Type II callus was transferred to B5O containing 150 mg/L Timentin plus 10 mg/L PPT and cultures were transferred to fresh medium every three weeks. Meristemoid/embryo development usually occured after one or two periods on B5O medium and were seen from about 14-16 weeks total culture time.

Plantlet development from embryos was slow and required a further 3 months in tissue culture before shoot and root growth was sufficient to ensure successful transplantation to soil.

If the initial pH of the medium was 5.8 and MES was omitted, the pH of poppy cultures rapidly rose to pH 8.0 or higher. Fresh agar-solidified B5-based medium adjusted to pH 5.6 rose to pH>6.4 in the immediate area around type II callus within 30 mins. The inclusion of chlorophenol red in the medium was used to observe these localised increases in pH; the medium turns purple at pH6.4. The whole plate was pH>7 within 24 h. At the end of the culture period pH values were measured at 8.7. This rapid rise in pH resulted in very poor growth which is not compensated for by frequent

changes of medium. The rapid rise was significantly delayed even by 2.5 mM MES, but 10 mM MES was preferred to adequately buffer the medium and support improved growth over the 3 week subculture period.

The identification and cloning of genes for cytochrome P-450 reductase enzymes now provides means by which the pathway of alkaloid metabolism can be regulated, specifically by alleviating the rate limiting steps which rely on cytochrome P-450. This in turn provides means of obtaining poppy plants with increased yield of alkaloids.

However, there will be instances where it may be preferable to manipulate the alkaloid metabolism of a poppy plant by suppression of genes encoding the P-450 reductases. The expression in the poppy of the cDNA encoding a P-450 reductase enzyme or part thereof, in an antisense orientation can be used to achieve this such that the expression directs the inhibition of the endogenous cytochrome P-450 reductase gene or homologues. In addition, the cDNA encoding the P-450 reductase enzyme or part thereof could be expressed in the sense orientation to direct the co-suppression of the endogenous cytochrome P-450 reductase gene or homologues. Furthermore, the cloned cDNA sequence can be used to design ribozyme sequences such as the hammerhead or hairpin ribozymes that can be used to suppress the target gene by inactivation of the endogenous cytochrome P-450 reductase gene mRNA. The genes encoding the sense, antisense or ribozymes can be delivered as transgenes stably integrated into the poppy genome or transiently in the form of a viral vector.

Although the invention has been described with reference to specific embodiments, modifications that are within the knowledge of those skilled in the art are also contemplated as being within the scope of the present invention.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS

1. An isolated and purified polynucleotide encoding a cytochrome P-450 reductase enzyme from an alkaloid poppy plant, or a variant, fragment or analog thereof.

2. A polynucleotide according to claim 1, selected from the group consisting of genomic DNA, cDNA, or synthetic DNA.

3. A polynucleotide according to claim 1 or claim 2, selected from the group consisting of:

(a) the polynucleotide sequences set out in Figures 6, 9 or 10; and

(b) complementary sequences of the polynucleotide sequences which hybridize under stringent conditions to the polynucleotide sequences defined in (a).

4. A polynucleotide according to any one of the preceding claims, lacking the native leader sequences or any of the 5' or 3' untranslated regions of the polynucleotide.

5. A polynucleotide according to claim 4, wherein the native leader sequences or any of the 5' or 3' untranslated regions are replaced with exogenous control/regulatory sequences which regulate optimised/enhanced expression of the polynucleotide in an expression system.

6. A polynucleotide according to any one of the preceding claims which encodes cytochrome P-450 reductase enzyme of *Papaver somniferum or Eschscholzia californica.*

7. A polynucleotide according to claim 6 which encodes cytochrome P-450 reductase enzyme of *Papaver somniferum.*

8. A polynucleotide according to claim 6 which encodes cytochrome P-450 reductase enzyme of *Eschscholzia californica.*

9. A polynticleotide according to any one of claims 2 to 8, which is a synthetic polynucleotide comprising one or more codons preferred for expression in plant cells.

10. An isolated and purified polynucleotide which codes for prokaryotic or eukaryotic expression of a cytochrome P-450 reductase enzyme from an alkaloid poppy plant, or a variant, analog or fragment thereof, wherein the polynucleotide is expressed in an environment selected from the group consisting of the extracellular environment, an intracellular membranous compartment, intracellular cytoplasmic compartment or combinations thereof.

11. A polynucleotide according to claim 10, comprising a nucleotide sequence which directs expression of the cytochrome P-450 reductase enzyme with respect to a particular cellular compartment or the extracellular environment.

12. An isolated and purified polynucleotide having a sequence which is complementary to all or part of the sequence of a polynucleotide according to any one of claims 1 to 11.

13. A recombinant DNA construct comprising the polynucleotide according to any one of claims 1 to 12.

14. A DNA construct according to claim 13, which is a viral or plasmid vector.

15. A DNA construct according to claim 13 or claim 14, capable of directing prokaryotic or eukaryotic expression of the polynucleotide encoding a cytochrome P-450 reductase enzyme.

16. A DNA construct according to any one of claims 13 to 15, comprising a promoter suitable to control the expression of the polynucleotide.

17. A DNA construct according to claim 16, wherein the promoter is endogenous.

18. A DNA construct according to claim 16, wherein the promoter is derived from cauliflower mosaic virus or subterranean clover mosaic virus.

19. An isolated and purified cytochrome P-450 reductase enzyme, being a product of prokaryotic or eukaryotic expression of the polynucleotide of any one of claims 1 to 11 or a DNA construct of any one of claims 13 to 18.

20. An enzyme according to claim 19, being a product of yeast cell expression.

21. An enzyme according to claim 19, being a product of bacterial cell expression.

22. An enzyme according to claim 19, being a product of animal cell expression.

23. An enzyme according to claim 22, being a product of insect cell expression,

24. An enzyme according to claim 19, being a product of plant cell expression.

25. An enzyme according to claim 24, wherein the plant cell is an alkaloid poppy plant cell.

26. An enzyme according to claim 25, wherein the alkaloid poppy *is Papaver somniferum*

27. An enzyme according to claim 25, wherein the alkaloid poppy is *Eschscholzia californica.*

28. An enzyme according to any one of claims 19 to 27, which is a variant incorporating amino acid deletions, substitutions, additions or combinations thereof, wherein the variant retains one or more of the biological properties of cytochrome P-450

reductase enzyme.

29. A cell transformed or transfected with a polynucleotide according to any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18.

30. A cell according to claim 29, which is a plant cell.

31. A cell according to claim 30, wherein the plant cell is derived from an alkaloid poppy plant.

32. A cell according to claim 31, wherein the poppy plant *is Papaver somniferum*

33. A cell according to claim 31, wherein the poppy plant is *Eschscholzia californica.*

34. A cell according to claim 29, which is a bacterial cell.

35. A cell according to claim 29, which is an animal cell.

36. A cell according to claim 29, which is a yeast cell.

37. A callus transformed or transfected with a polynucleotide according to any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18.

38. A plant transformed or transfected with a polynucleotide according to any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18, wherein the plant exhibits altered expression of the cytochrome P-450 reductase enzyme.

39. A plant according to claim 38, wherein the altered expression is overexpression of the cytochrome P-450 reductase enzyme.

40. A plant according to claim 38, wherein the altered expression is reduced expression of the cytochrome P-450 reductase enzyme.

41. A plant according to any one of claims 38 to 40, which is an alkaloid poppy plant.

42. A plant according to claim 41, wherein the plant has a higher or different alkaloid content when compared to a plant which has not been so transformed or transfected.

43. A plant according to claim 41 or claim 42, wherein the alkaloid poppy plant is *Papaver somniferum.*

44. A plant according to claim 41 or claim 42, wherein the alkaloid poppy plant is *Eschscholzia californica.*

45. A method for preparing plants which overexpress a cytochrome P-450 reductase enzyme, comprising transfecting or transforming a plant cell, a plant part or a plant with the polynucleotide according to any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18.

46. A method according to claim 45, wherein the plant is an alkaloid poppy plant.

47. A method according to claim 46, wherein the poppy plant *is Papaver somniferum.*

48. A method according to claim 46, wherein the poppy plant *is Eschscholzia californica.*

49. A method of altering the yield or type of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, or with a polynucleotide which binds under stringent conditions to the polynucleotide encoding said enzyme.

50. A method of increasing the yield of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, wherein the enzyme is overexpressed in said plant.

51. A method of altering type or blend of alkaloid in a plant comprising transforming or transfecting a plant cell, a plant part or a plant with a polynucleotide encoding a cytochrome P-450 reductase enzyme or a variant, analog or fragment thereof, or with a polynucleotide which binds under stringent conditions to the polynucleotide encoding said enzyme.

52. A stand of stably reproducing alkaloid poppies transformed or transfected with a polynucleotide according to any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18, having altered expression of the cytochrome P-450 reductase enzyme.

53. A stand of stably reproducing alkaloid poppies transformed or transfected with a polynucleotide according to any one of claims 1 to 11 or a DNA construct according to any one of claims 13 to 18, having a higher or different alkaloid content when compared to a plant which has not been so transformed or transfected.

54. A stand of stably reproducing alkaloid poppies according to claim 52 or claim 53, wherein the alkaloid poppy is *Papaver somniferum.*

55. Straw of stably reproducing poppies according to any one of claims 52 to 54, having a higher or different alkaloid content when compared to the straw obtained from an alkaloid poppy which has not been transformed or transfected.

56. A concentrate of straw according to claim 55, having a higher or different alkaloid content when compared to the concentrate of straw obtained from an alkaloid poppy which has not been transformed or transfected.

57. An alkaloid when isolated from the straw according to any one of claims 52 to 55 or the concentrate according to claim 56.

58. An alkaloid according to claim 57 selected from the group consisting of morphine, codeine, oripavine and thebaine.

59. A method for the production of poppy plant alkaloids, comprising the steps of;

a) harvesting capsules of an alkaloid poppy plant transformed or transfected with a polynucleotide according to any one of claims 1 to 12 or a DNA construct according to any one of claims 13 to 18, to produce a straw where the poppy plant is such a plant that the straw has a higher or different alkaloid content when compared to the straw obtained from a poppy plant which has not been transformed or transfected.

b) chemically extracting the alkaloids from the straw.

60. A method for the production of poppy alkaloids comprising the steps of;

a) collecting and drying the latex of the immature capsules of an alkaloid poppy plant transformed or transfected with a polynucleotide according to any one of claims 1 to 12 or a DNA construct according to any one of claims 13 to 18, to produce opium where the poppy plant is such a plant that the opium has a higher or different alkaloid content when compared to the opium obtained from a poppy plant which has not been transformed or transfected.

b) chemically extracting the alkaloids from the opium.

61. A method according to claim 59 or claim 60, wherein the alkaloid is selected from the group consisting of morphine, codeine, oripavine and thebaine.

ABSTRACT

The present invention relates to production of alkaloids from poppy plants and in particular togenes encoding enzymes in the alkaloid pathway, to proteins encoded by the gens, to plants transformed or transfected with the genes and to methods of altering alkaloid content or blend of poppy plants.

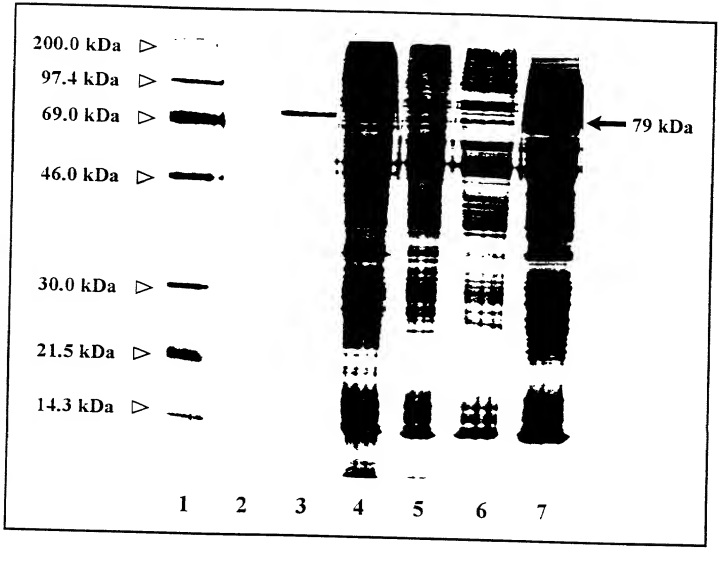
200.0 kDa
97.4 kDa
69.0 kDa
46.0 kDa
30.0 kDa
21.5 kDa
14.3 kDa
79 kDa
1
2
3
4
5
6
7

Figure 1.

Peptide 1 KVTIFFGTQK

Peptide 2 KVVDLDDYAADDDEFEEK
E

Peptide 3 KWFTEVAK
D

Peptide 4 KVVDEIIVEK

Peptide 5 KYADLLNFPK

Peptide 6 KAALHALAK

Peptide 7 KDVHRTLHTIVQEQGSLDSSK

Figure 2.

```
Arabidopsis thaliana   ..350 GSPLES-AVPPPFPGPCT
Catharanthus roseus    ..389 GTPLAGSSLPPPFP-PCT
Helianthus tuberosus   ..331 GTPLGGPTLQPPFP-PCT
Vigna radiata          ..366 GTSLGG-SLLPPFPGPCS
Vicia sativa           ..367 GTSLGG-SLLPPFPGPCT
                             *. *   .. **** **.

LGTGLARYADLLNPPRKSALVALAAYATEPSEAEKLKHLTSPDGKD
LRTALTRYADLLNTPKKSALLALAAYASDPNEADRLKYLASPAGKD
LRKALTNYADLLSSPKKSTLLALAAHASDATEADRLQFLASREGKD
LRTALARYADLLNPPRKAALLALATHASEPS-DERLKFLSSPQGKD
VRTALACYADLLNPPRKAAIVALAAHASEPSEAERLKFLSSPQGKD
.   *  *****  *.*   .***  *.        *    ** ***

EYSQWIVASQRSLLEVMAAFPSAKPPLGVFFAAIAPRLQPRYYSIS
EYAQSLVANQRSLLEVMAEFPSAKPPLGVFFAAIAPRLQPRFYSIS
EYAEWIVANQRSLLEVMEAFPSAKPPLGVFFAAIAPRLQPRYYSIS
EYSKWVVGSQRSLVEVMAEFPSAKPPLGVFFAAIAPRLQPRYYSIS
EYSKWVVGSQRSLLEVMADFPSAKPPLGVFFAAIAPRLQPRYYSIS
**.  .* ****.***  **********************.****

SCQDWAPSRVHVTSALVYGPTPTGRIHKGVCSTWMKNAVP 498..
SSPRMAPSRIHVTCALVYEKTPGGRIHKGVCSTWMKNAIP 537..
SSPKMVPNRIHVTCALVYEKTPGGRIHKGICSTWMKNAVP 479..
SSPRFAPQRVHVTCALVYGPTPTGRIHKGVCSTWMKNAIP 513..
SSPRPAPQRVHVTCALVEGPTPTGRIHKGVCSTWMKSATP 515..
      *.    * *.***.***   ** ******.****** * *
```

Figure 3.

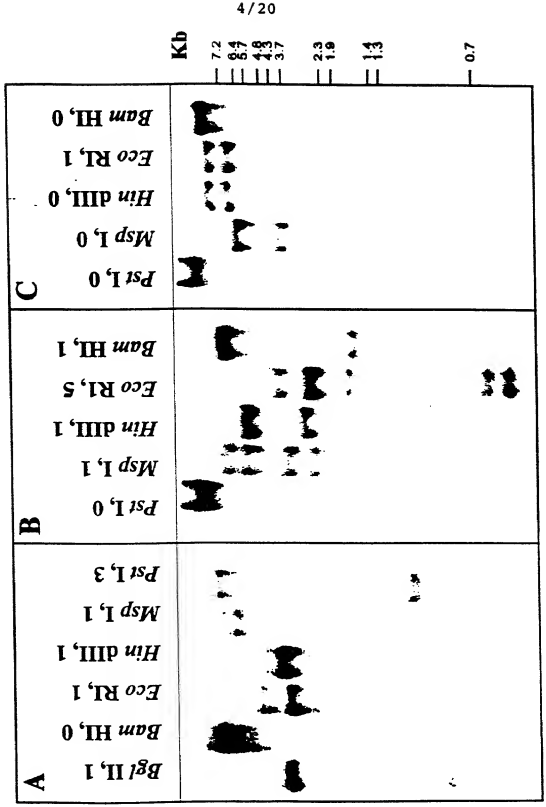
Kb
7.2
6.4
4.8
4.3
3.7
2.3
1.9
1.3
0.7
A
Bgl II, 1
Bam HI, 0
Eco RI, 1
Hin dIII, 1
Msp I, 1
Pst I, 3
B
Pst I, 0
Msp I, 1
Hin dIII, 1
Eco R1, 5
Bam HI, 1
C
Pst I, 0
Msp I, 0
Hin dIII, 0
Eco RI, 1
Bam HI, 0

Figure 4.

```
1                                                                   50
MEQTAVKVSL FDLFSSILNG KLDPSNFSSD SSAAILIENR EILMILTTAI
MGSNNLANSI ESMLG.ISIG ....SEYISD P.........  .IFIMVTTVA
*     *       *  *     *  **                  *   **
51                                                                 100
AVFIGCGFLY VWRRSSNKSS KIVETQKLIV EKEPE.PEVD DGKKKVTIFF
SMLIGFGFFV CMK.SSSSQS KPIETYKPII DKEEEEIEVD PGKIKLTIFF
   **  **        **   * *  ** * *   ***  *   ** * ****
101                                                                150
GTQTGTAEGF AKALAEEAKA RYEKAIFKVI DLDDYGADDD EFEEKLKKET
GTQTGTAEGF AKALAEEIKA KYKKAVVKVV DLDDYAAEDD QYEEKLKKES
********** ******* **  * **  **  ***** * **   *******
151                                                                200
IALFFLATYG DGEPTDNAAR FYKWFTEGKE REMWLQNLQF GVFGLGNRQY
LVFFMVATYG DGEPTDNAAR FYKWFTQEHE RGEWLQQLTY GVFGLGNRQY
   *  **** ********** ******   * *  *** *   **********
201                                                                250
EHFNKVAKEV DEILTEQGGK RIVPVGLGDD DQCIEDDFTA WRELVWPELD
EHFNKIAVDV DEQLGKQGAK RIVQVGLGDD DQCIEDDFTA WRELLWTELD
***** *  * ** *  ** * *** ****** ********** **** * ***
251                                                                300
QLLLDESDKT SVSTPYTAIV PEYRVVFHDA TDASLQDKNW SNANGVTVYD
QLLKDEDAAP SVATPYIATV PEYRVVIHET TVAALDDKHI NTANGDVAFD
*** **     ** *** * * ****** *   * * * **     ***   *
301                                                                350
VQHPCRANVV VKKELHTPVS DRSCIHLEFD ISGTGLTYET GDHVGVYSEN
ILHPCRTIVA QQRELHKPKS DRSCIHLEFD ISGSSLTYET GDHVGVYAEN
  ****  *     *** * * ********** ***  ***** ******* **
351                                                                400
CVEVVEEAER LLGYSSDTVF SIHVDKEDGS PISGSALAPP FPTPCTLRTA
CDETVEEAGK LLGQPLDLLF SIHTDKEDGS PQGSS..LPP FPGPCTLRSA
* * ****   ***   *  * *** ****** *   *   ** ** ***** *
401                                                                450
LTRYADLLNS PKKAALHALA AYASDPKEAE RLRYLASPAG KDEYAQWIVA
LARYADLLNP PRKASLIALS AHASVPSEAE RLRFLSSPLG KNEYSKWVVG
* *******  * ** * **  * ** * *** *** * ** * * **  * *
451                                                                500
SQRSLLVVMA EFPSAKAPIG VFFAAVAPRL LPRYYSISSS NRMVPSRIHV
SQRSLLEIMA EFPSAKPPLG VFFAAVAPRL PPRYYSISSS PKFAPSRIHV
******  ** ****** * * **********  **********      ******
501                                                                550
TCALVHEKTP AGRVHKGVCS TWMKNSVSLE ENHDCSSWAP IFVRQSNFKL
TCALVYGQSP TGRFHRGVCS TWMKHAVPQD S......WAP IFVRTSNFKL
*****    *  ** * **** ****  *            *** **** *****
551                                                                600
PADSTVPIIM IGPGTGLAPF RGFMQERLAL KNSGVELGPA ILFFGCRNRQ
PADPSTPIIM VGPGTGLAPF RGFLQERMAL KENGAQLGPA VLFFGCRNRN
***   ****  ********** *** *** ** *  *  ****  ********
601                                                                650
MDYIYEEELN NFVKEGAISE VVVAFSREGA TKEYVQHKMA EKASYIWEMI
MDFIYEDELN NFVERGVISE LVIAFSREGE KKEYVQHKMM EKATDVWNVI
** *** *** ***  * ***  * ******   ********  ***   * *
651                                                                700
SQGAYLYVCG DAKGMARDVH RTLHTIAQEQ GSLDNSKTES LVKNLQMDGR
SGDGYLYVCG DAKGMARDVH RTLHTIAQEQ GSMESSAAEA AVKKLQVEER
*   ****** ********** ********** **   *  *   ** **   *
701
YLRDVW
YLRDVW
******
```

Figure 5.

CGGCACGAGCTTGTTAGTATCTTCTAGGGTTTGAAAAGAAGCACAGGGAGAAGCAAAAGTCGAATCTACTTGAAATACAT
TCGATTGCTTCTCTCTGTTTAAGCTTCAGAGTCTCTGCTAATTATGGGTTCGAATAATTTAGCTAATTCGATTGAATCGA
TGTTAGGAATATCAATAGGATCAGAATATATTTCTGACCCAATTTTCATTATGGTCACAACTGTAGCTTCAATGCTGATT
GGATTTGGTTTCTTCGCATGTATGAAATCTTCGTCTTCTCAATCAAAACCTATTGAAACTTATAAACCAATAATTGATAA
AGAAGAAGAGGAGATTGAAGTTGATCCTGGTAAAATTAAGCTCACTATATTTTTTGGTACTCAGACTGGTACTGCTGAAG
GATTTGCTAAGGCATTGGCAGAAGAAATTAAGGCAAAGTACAAGAAAGCAGTTGTTAAAGTAGTTGACCTGGATGACTAT
GCAGCCGAGGATGATCAATATGAAGAGAAATTAAAGAAAGAGTCTTTGGTGTTTTTCATGGTAGCCACTTATGGTGATGG
TGAGCCAACTGACAATGCTGCGAGATTTTACAAATGGTTCACTCAGGAACATGAAAGGGGAGAGTGGCTTCAGCAACTAA
CTTATGGTGTTTTTGGTTTGGGTAACCGTCAATACGAGCATTTCAACAAGATCGCGGTAGATGTGGATGAGCAACTCGGT
AAACAAGGTGCAAAGCGCATTGTTCAAGTGGGGCTCGGTGACGATGATCAATGCATTGAAGATGATTTTACTGCTTGGCG
AGAATTGTTGTGGACTGAATTGGATCAGTTGCTCAAAGATGAGGATGCTGCTCCTTCAGTGGCTACACCGTATATTGCTA
CTGTTCCTGAATACAGGGTAGTGATTCACGAAACTACGGTCGCGGCTCTGGATGATAAACACATAAATACTGCTAACGGC
GATGTTGCATTTGATATTCTCCATCCTTGCAGAACCATTGTTGCTCAACAAAGAGAGCTCCACAAACCCAAGTCTGATAG
ATCCTGTATACATCTGGAGTTCGACATATCAGGCTCTTCCCTTACATATGAGACTGGAGATCATGTTGGTGTTTATGCTG
AGAACTGCGATGAAACTGTCGAGGAAGCAGGGAAGCTGTTGGGTCAACCCCTGGATTTGCTGTTTTCAATTCACACGGAT
AAAGAAGACGGGTCACCCCAGGGAAGCTCATTACCACCTCCTTTCCCAGGTCCTTGCACCTTACGATCTGCCCTAGCACG
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AGAGATTGCGCTTTTTGTCATCACCTCTGGGAAAGAATGAGTATTCAAAATGGGTAGTTGGAAGTCAGAGGAGTCTTTTG
GAGATCATGGCCGAGTTTCCATCAGCAAAACCCCCTCTTGGTGTTTTCTTTGCTGCAGTAGCCCCTCGCTTACCGCCTCG
ATACTATTCTATCTCATCCTCTCCTAAGTTTGCTCCCTCAAGAATTCATGTGACGTGTGCTTTAGTATATGGTCAAAGCC
CTACCGGAAGGGTTCACCGAGGAGTGTGTTCGACATGGATGAAGCATGCAGTTCCTCAGGATAGCTGGGCTCCTATTTTT
GTTCGAACGTCAAACTTCAAGTTTACCAGCTGACCCCTCAACTCCAATTATCATGGTGGGACCTGGTACAGGGTTAGCTCC
TTTCAGAGGATTTCTGCAGGAAAGAATGGCCCCTCAAGGAAAATGGTGCTCAACTTGGCCCAGCAGTGCTCTTTTTCGGAT
GTAGGAATCGTAATATGGACTTCATTTATGAAGACGAACTAAACAACTTCGTGGAACGAGGAGTAATTTCGGAGCTAGTT
ATTGCCTTTTCACGTGAAGGGGAAAAGAAGGAATATGTTCAACATAAGATGATGGAGAAAGCAACGGATGTATGGAATGT
GATATCAGGGGACGGTTATCTCTATGTGTGTGGTGATGCCAAGGGAATGGCCAGAGATGTCCATCGCACGTTGCATACCA
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AGAGATGTCTGGTGATCGAATGTAGCTTGCCAAGTCCCCTTTTCTTGGCTGGTCTGTTTATGGTTTCTATTATATTATTG
ATCCTCCTCTGAAAATCCCAAGCACTTCCAGACATCCCTCGATTCTTCCTCCAGTGGTTCCAAATCGAAGCTCGGTATAA
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GAACAATGTTACAGGCAAAACTGTGTTTGCTTAATATAAATTTCACACCATGGGTGTGGACAACACTGAAACAGTATTAG
CTATACCAACAAAGTTATGCAAGAAACACAAACTAGTTAGATCTTCTCTTTGGATTGATTACTGTAAGTTCTAACCAGA
TGATAGATTGTACTTAAAGATTCTTGTTTTCTTATGGCTACCGAGAGGAGTATATTAATGCATTTAGAGTTTTGAGAAAAAAAAAAAAA

Figure 6a

TTCTTCTTCCAATCGCATTCGAGAAAATTCAATCATCTTCAACTTCAGGAAGAAGAATCATCAGAAACACTGAAGCTCAT
CATCATCCTTGAAACTTATCGTCTTTGTTTGACCTTTTGAAAAACTATGGAACAAACTGCGGTTAAAGTCTCTTTGTTTG
ATCTATTTTCTTCGATACTTAATGGAAAGTTGGATCCGTCGAACTTTTCTTCAGATTCAAGTGCTGCTATTTTGATTGAA
AATCGTGAGATTTTAATGATCTTAACAACTGCTATTGCTGTTTTTATCGGTTGTGGTTTTCTCTACGTTTGGAGAAGATC
TTCAAATAAGTCGAGTAAAATTGTTGAAACTCAGAAATTGATCGTTGAAAAGGAACCAGAACCTGAAGTTGATGATGGAA
AGAAGAAGGTTACTATCTTCTTTGGTACTCAAACTGGTACAGCTGAAGGATTCGCAAAGGCACTTGCTGAAGAAGCAAAA
GCAAGATATGAAAAGGCAATCTTTAAAGTGATTGATCTGGATGATTACGGAGCAGATGATGATGAATTCGAAGAGAAATT
GAAAAAGGAAACTATAGCTCTTTTCTTTTTGGCTACCTATGGAGATGGTGAACCTACAGATAATGCTGCAAGATTTTATA
AATGGTTCACAGAGGGgAGAGAGGGAAATGTGGCTCCAGAATCTTCAATTTGGTGTCTTCGGTCTAGGCAATAGACAGTA
TGAGCATTTCAATAAGGTGGCAAAGGAGGTGGACGAGATACTCACTGAACAGGGTGGGAAGCGTATTGTTCCCGTGGGTC
TAGGAGATGATGATCAATGCATAGAAGATGATTTCACTGCGTGGCGGGAGTTGGTATGGCCTGAATTGGATCAGTTGCTC
CTTGATGAAAGTGATAAAACATCTGTTTCTACTCCTTACACTGCCATCGTACCAGAATACAGGGTAGTATTCCATGATGC
TACTGATGCATCACTACAAGACAAAAACTGGAGCAATGCAAATGGCTACACTGTTTACGACGTTCAACACCCATGCAGAG
CCAATGTCGTTGTAAAGAAGGAGCTTCACACTCCAGTATCTGATCGTTCTTGTATTCATCTGGAATTTGACATTTCTGGC
ACTGGGCTCACGTATGAAACAGGAGACCATGTCGGTGTTTACTCTGAGAATTGTGTTGAAGTTGTCGAGGAAGCAGAGAG
GCTATTGGGTTACTCATCAGACACCGTTTTTTCAATCCATGTCGATAAAGAGGACGGCTCACCCATTAGTGGAAGCGCTC
TAGCTCCTCCTTTTCCAACTCCCTGCACTCTAAGAACAGCACTAACACGATACGCTGATCTGTTGAATTCTCCCAAGAAG
GCTGCTCTGCATGCTTTGGCTGCTTATGCATCCGATCCAAAGGAAGCGGAGCGACTAAGGTATCTTGCGTCTCCTGCTGG
GAAGGACGAATACGCCCAGTGGATAGTAGCTAGTCAGAGAAGTCTGCTAGTGGTCATGGCTGAATTCCCATCAGCAAAGG
CTCCAATTGGGGTTTTCTTTGCAGCAGTAGCTCCTCGCTTGCTGCCAAGATACTATTCTATTTCATCTTCCAATAGGATG
GTACCATCTAGGATTCATGTCACATGTGCATTGGTGCATGAAAAAACACCGGCAGGTCGGGTTCACAAAGGAGTGTGTTC
AACCTGGATGAAGAATTCTGTGTCTTTGGAAGAAAACCATGATTGCAGCAGCTGGGCACCAATCTTTGTCAGGCAATCCA
ACTTCAAACTTCCTGCTGATTCTACAGTACCAATTATAATGATTGGTCCTGGGACTGGATTAGCTCCCTTTAGGGATTC
ATGCAGGAGCGATTAGCTCTGAAGAATTCTGGTGTAGAATTGGGACCCGCTATCCTCTTCTTTGGATGCAGAAACAGACA
GATGGATTACATATATGAAGAGGAGCTAAACAACTTTGTGAAAGAGGGAGCTATCTCCGAAGTTGTTGTTGCTTTCTCAC
GTGAGGGAGCTACCAAGGAATACGTACAACATAAAATGGCGGAGAAGGCTTCCTACATCTGGGAAATGATCTCTCAAGGT
GCTTATCTTTATGTATGTGGTGATGCCAAGGGCATGGCTAGAGACGTACATCGAACTCTCCACACCATTGCCCAGGAACA
GGGATCTTTGGACAACTCGAAGACCGAAAGCTTGGTGAAGAATCTACAGATGGATGGAAGGTATCTACGTGATGTGTGGT
GATTGATTTTTTCAGCACGGTTACAATCTAGCTTCATCAAAGAACGCGCTTGAGAAGCATAAATCTTAGTTGCAGAGATG
TTGATTTCAGAAGAAATGCTTTATATACTTGAGGTAGCGGACATTAATCCTTTTCTCTCTCTCTAAACTGTTAATCCTGT
AAAAAAGGGATTGCTGTTTGTGTTTGCTCGCAATCAATTAAGTTATATTCTTTGGTCTATGGCATTCGTTAGACAAATAT
ATTAACGAGTTTGTCCGTTATATATGACATATGAAACAAAAGAACTTCTGTTTGGAGGAAGAGAAAAAAAAAAAAAA

Figure 6b.

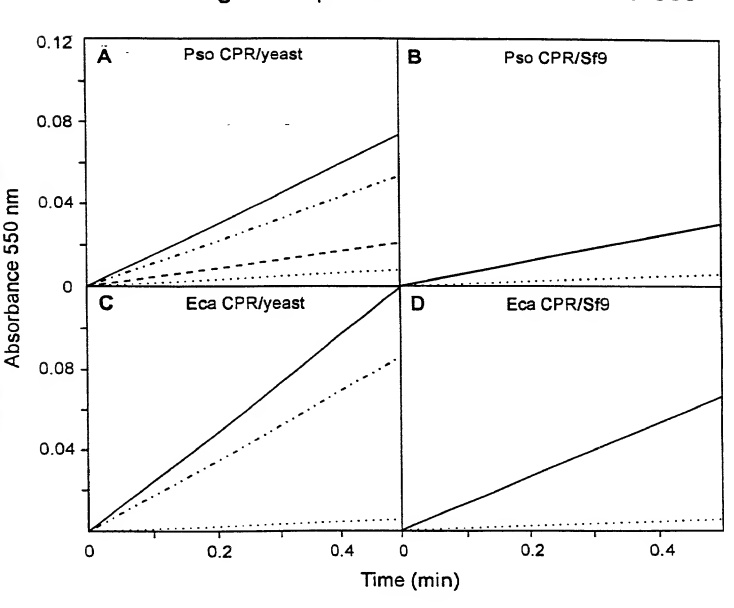
Heterologous Expression of P-450 Reductases
A
Pso CPR/yeast
B
Pso CPR/Sf9
C
Eca CPR/yeast
D
Eca CPR/Sf9
Absorbance 550 nm
0.12
0.08
0.04
0
0.08
0.04
0
0.2
0.4
0
0.2
0.4
Time (min)

Figure 7.

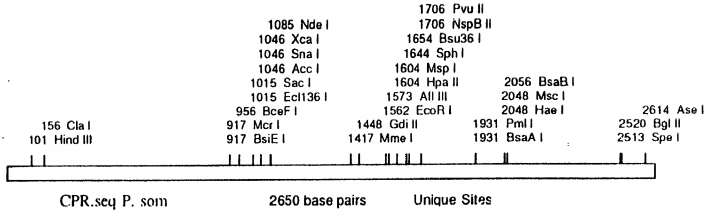

Figure 8a.

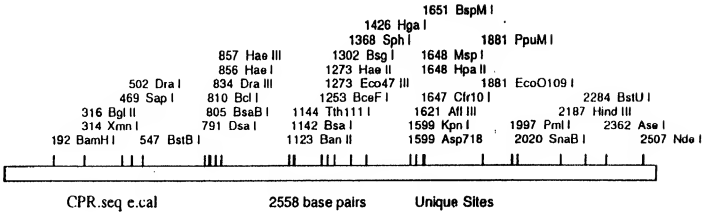

Figure 8b.

```
1/1                                                                 31/11
CGG CAC GAG CTT GTT AGT ATC TTC TAG GGT TTG AAA AGA AGC ACA GGG AGA AGC AAA AGT
R   H   E   L   V   S   I   F   *   G   L   K   R   S   T   G   R   S   K   S
61/21                                                               91/31
CGA ATC TAC TTG AAA TAC ATT CGA TTG CTT CTC TCT GTT TAA GCT TCA GAG TCT CTG CTA
R   I   Y   L   K   Y   I   R   L   L   L   S   V   *   A   S   E   S   L   L
121/41                                                              151/51
ATT ATG GGT TCG AAT AAT TTA GCT AAT TCG ATT GAA TCG ATG TTA GGA ATA TCA ATA GGA
I   M   G   S   N   N   L   A   N   S   I   E   S   M   L   G   I   S   I   G
181/61                                                              211/71
TCA GAA TAT ATT TCT GAC CCA ATT TTC ATT ATG GTC ACA ACT GTA GCT TCA ATG CTG ATT
S   E   Y   I   S   D   P   I   F   I   M   V   T   T   V   A   S   M   L   I
241/81                                                              271/91
GGA TTT GGT TTC TTC GCA TGT ATG AAA TCT TCG TCT TCT CAA TCA AAA CCT ATT GAA ACT
G   F   G   F   F   A   C   M   K   S   S   S   S   Q   S   K   P   I   E   T
301/101                                                             331/111
TAT AAA CCA ATA ATT GAT AAA GAA GAA GAG GAG ATT GAA GTT GAT CCT GGT AAA ATT AAG
Y   K   P   I   I   D   K   E   E   E   E   I   E   V   D   P   G   K   I   K
361/121                                                             391/131
CTC ACT ATA TTT TTT GGT ACT CAG ACT GGT ACT GCT GAA GGA TTT GCT AAG GCA TTG GCA
L   T   I   F   F   G   T   Q   T   G   T   A   E   G   F   A   K   A   L   A
421/141                                                             451/151
GAA GAA ATT AAG GCA AAG TAC AAG AAA GCA GTT GTT AAA GTA GTT GAC CTG GAT GAC TAT
E   E   I   K   A   K   Y   K   K   A   V   V   K   V   V   D   L   D   D   Y
481/161                                                             511/171
GCA GCC GAG GAT GAT CAA TAT GAA GAG AAA TTA AAG AAA GAG TCT TTG GTG TTT TTC ATG
A   A   E   D   D   Q   Y   E   E   K   L   K   K   E   S   L   V   F   F   M
541/181                                                             571/191
GTA GCC ACT TAT GGT GAT GGT GAG CCA ACT GAC AAT GCT GCG AGA TTT TAC AAA TGG TTC
V   A   T   Y   G   D   G   E   P   T   D   N   A   A   R   F   Y   K   W   F
601/201                                                             631/211
ACT CAG GAA CAT GAA AGG GGA GAG TGG CTT CAG CAA CTA ACT TAT GGT GTT TTT GGT TTG
T   Q   E   H   E   R   G   E   W   L   Q   Q   L   T   Y   G   V   F   G   L
661/221                                                             691/231
GGT AAC CGT CAA TAC GAG CAT TTC AAC AAG ATC GCG GTA GAT GTG GAT GAG CAA CTC GGT
G   N   R   Q   Y   E   H   F   N   K   I   A   V   D   V   D   E   Q   L   G
721/241                                                             751/251
AAA CAA GGT GCA AAG CGC ATT GTT CAA GTG GGG CTC GGT GAC GAT GAT CAA TGC ATT GAA
K   Q   G   A   K   R   I   V   Q   V   G   L   G   D   D   D   Q   C   I   E
781/261                                                             811/271
GAT GAT TTT ACT GCT TGG CGA GAA TTG TTG TGG ACT GAA TTG GAT CAG TTG CTC AAA GAT
D   D   F   T   A   W   R   E   L   L   W   T   E   L   D   Q   L   L   K   D
841/281                                                             871/291
GAG GAT GCT GCT CCT TCA GTG GCT ACA CCG TAT ATT GCT ACT GTT CCT GAA TAC AGG GTA
E   D   A   A   P   S   V   A   T   P   Y   I   A   T   V   P   E   Y   R   V
901/301                                                             931/311
GTG ATT CAC GAA ACT ACG GTC GCG GCT CTG GAT GAT AAA CAC ATA AAT ACT GCT AAC GGC
V   I   H   E   T   T   V   A   A   L   D   D   K   H   I   N   T   A   N   G
961/321                                                             991/331
GAT GTT GCA TTT GAT ATT CTC CAT CCT TGC AGA ACC ATT GTT GCT CAA CAA AGA GAG CTC
D   V   A   F   D   I   L   H   P   C   R   T   I   V   A   Q   Q   R   E   L
1021/341                                                            1051/351
```

Figure 9a.

```
CAC AAA CCC AAG TCT GAT AGA TCC TGT ATA CAT CTG GAG TTC GAC ATA TCA GGC TCT TCC
H   K   P   K   S   D   R   S   C   I   H   L   E   F   D   I   S   G   S   S
1081/361                                1111/371
CTT ACA TAT GAG ACT GGA GAT CAT GTT GGT GTT TAT GCT GAG AAC TGC GAT GAA ACT GTC
L   T   Y   E   T   G   D   H   V   G   V   Y   A   E   N   C   D   E   T   V
1141/381                                1171/391
GAG GAA GCA GGG AAG CTG TTG GGT CAA CCC CTG GAT TTG CTG TTT TCA ATT CAC ACG GAT
E   E   A   G   K   L   L   G   Q   P   L   D   L   L   F   S   I   H   T   D
1201/401                                1231/411
AAA GAA GAC GGG TCA CCC CAG GGA AGC TCA TTA CCA CCT CCT TTC CCA GGT CCT TGC ACC
K   E   D   G   S   P   Q   G   S   S   L   P   P   P   F   P   G   P   C   T
1261/421                                1291/431
TTA CGA TCT GCC CTA GCA CGC TAT GCT GAT CTT TTG AAT CCT CCT AGA AAG GCT TCT CTG
L   R   S   A   L   A   R   Y   A   D   L   L   N   P   P   R   K   A   S   L
1321/441                                1351/451
ATT GCT CTG TCC GCT CAT GCA TCT GTA CCC AGT GAA GCA GAG AGA TTG CGC TTT TTG TCA
I   A   L   S   A   H   A   S   V   P   S   E   A   E   R   L   R   F   L   S
1381/461                                1411/471
TCA CCT CTG GGA AAG AAT GAG TAT TCA AAA TGG GTA GTT GGA AGT CAG AGG AGT CTT TTG
S   P   L   G   K   N   E   Y   S   K   W   V   V   G   S   Q   R   S   L   L
1441/481                                1471/491
GAG ATC ATG GCC GAG TTT CCA TCA GCA AAA CCC CCT CTT GGT GTT TTC TTT GCT GCA GTA
E   I   M   A   E   F   P   S   A   K   P   P   L   G   V   F   F   A   A   V
1501/501                                1531/511
GCC CCT CGC TTA CCG CCT CGA TAC TAT TCT ATC TCA TCC TCT CCT AAG TTT GCT CCC TCA
A   P   R   L   P   P   R   Y   Y   S   I   S   S   S   P   K   F   A   P   S
1561/521                                1591/531
AGA ATT CAT GTG ACG TGT GCT TTA GTA TAT GGT CAA AGC CCT ACC GGA AGG GTT CAC CGA
R   I   H   V   T   C   A   L   V   Y   G   Q   S   P   T   G   R   V   H   R
1621/541                                1651/551
GGA GTG TGT TCG ACA TGG ATG AAG CAT GCA GTT CCT CAG GAT AGC TGG GCT CCT ATT TTT
G   V   C   S   T   W   M   K   H   A   V   P   Q   D   S   W   A   P   I   F
1681/561                                1711/571
GTT CGA ACG TCA AAC TTC AAG TTA CCA GCT GAC CCC TCA ACT CCA ATT ATC ATG GTG GGA
V   R   T   S   N   F   K   L   P   A   D   P   S   T   P   I   I   M   V   G
1741/581                                1771/591
CCT GGT ACA GGG TTA GCT CCT TTC AGA GGA TTT CTG CAG GAA AGA ATG GCC CTC AAG GAA
P   G   T   G   L   A   P   F   R   G   F   L   Q   E   R   M   A   L   K   E
1801/601                                1831/611
AAT GGT GCT CAA CTT GGC CCA GCA GTG CTC TTT TTC GGA TGT AGG AAT CGT AAT ATG GAC
N   G   A   Q   L   G   P   A   V   L   F   F   G   C   R   N   R   N   M   D
1861/621                                1891/631
TTC ATT TAT GAA GAC GAA CTA AAC AAC TTC GTG GAA CGA GGA GTA ATT TCG GAG CTA GTT
F   I   Y   E   D   E   L   N   N   F   V   E   R   G   V   I   S   E   L   V
1921/641                                1951/651
ATT GCC TTT TCA CGT GAA GGG GAA AAG AAG GAA TAT GTT CAA CAT AAG ATG ATG GAG AAA
I   A   F   S   R   E   G   E   K   K   E   Y   V   Q   H   K   M   M   E   K
1981/661                                2011/671
GCA ACG GAT GTA TGG AAT GTG ATA TCA GGG GAC GGT TAT CTC TAT GTG TGT GGT GAT GCC
A   T   D   V   W   N   V   I   S   G   D   G   Y   L   Y   V   C   G   D   A
2041/681                                2071/691
```

Figure 9a (cont.).

```
AAG GGA ATG GCC AGA GAT GTC CAT CGC ACG TTG CAT ACC ATT GCC CAA GAA CAG GGA CCC
K   G   M   A   R   D   V   H   R   T   L   H   T   I   A   Q   E   Q   G   P
2101/701                                        2131/711
ATG GAA TCA TCT GCT GCC GAA GCT GCA GTA AAG AAA CTC CAA GTT GAA GAA CGA TAT CTA
M   E   S   S   A   A   E   A   A   V   K   K   L   Q   V   E   E   R   Y   L
2161/721                                        2191/731
AGA GAT GTC TGG TGA TCG AAT GTA GCT TGC CAA GTC CCC TTT TCT TGG CTG GTC TGT TTA
R   D   V   W   *   S   N   V   A   C   Q   V   P   F   S   W   L   V   C   L
2221/741                                        2251/751
TGG TTT CTA TTA TAT TAT TGA TCC TCC TCT GAA AAT CCC AAG CAC TTC CAG ACA TCC CTC
W   F   L   L   Y   Y   *   S   S   S   E   N   P   K   H   F   Q   T   S   L
2281/761                                        2311/771
GAT TCT TCC TCC AGT GGT TCC AAA TCG AAG CTC GGT ATA ATT GAG AGC AGT GCA ATT GTG
D   S   S   S   S   G   S   K   S   K   L   G   I   I   E   S   S   A   I   V
2341/781                                        2371/791
ACT ACA TGA GAA GCA AAC ATC GAA TAC CAT AGA ATT AGA AAG ATC AAA ATT CTC TTA TCA
T   T   *   E   A   N   I   E   Y   H   R   I   R   K   I   K   I   L   L   S
2401/801                                        2431/811
GAA CAA TGT TAC AGG CAA AAC TGT GTT TGC TTA ATA TAA ATT TCA CAC CAT GGG TGT GGA
E   Q   C   Y   R   Q   N   C   V   C   L   I   *   I   S   H   H   G   C   G
2461/821                                        2491/831
CAA CAC TGA AAC AGT ATT AGC TAT ACC AAC AAA GTT ATG CAA GGA AAC ACA AAC TAG TTA
Q   H   *   N   S   I   S   Y   T   N   K   V   M   Q   G   N   T   N   *   L
2521/841                                        2551/851
GAT CTT CTC TTT GGA TTG ATT ACT GTA AGT TCT AAC CAG ATG ATA GAT TGT ACT TAA AGA
D   L   L   F   G   L   I   T   V   S   S   N   Q   M   I   D   C   T   *   R
2581/861                                        2611/871
TTC TTG TTT TCT TAT GGC TAC CGA GAG GAG TAT ATT AAT GCA TTT AGA GTT TTG AGA AAA
F   L   F   S   Y   G   Y   R   E   E   Y   I   N   A   F   R   V   L   R   K
2641/881
AAA AAA AAA A
K   K   K
```

Figure 9a (cont.).

```
1/1                                                                 31/11
TTC TTC TTC CAA TCG CAT TCG AGA AAA TTC AAT CAT CTT CAA CTT CAG GAA GAA GAA TCA
F   F   F   Q   S   H   S   R   K   F   N   H   L   Q   L   Q   E   E   E   S
61/21                                                               91/31
TCA GAA ACA CTG AAG CTC ATC ATC ATC CTT GAA ACT TAT CGT CTT TGT TTG ACC TTT TGA
S   E   T   L   K   L   I   I   I   L   E   T   Y   R   L   C   L   T   F   *
121/41                                                              151/51
AAA ACT ATG GAA CAA ACT GCG GTT AAA GTC TCT TTG TTT GAT CTA TTT TCT TCG ATA CTT
K   T   M   E   Q   T   A   V   K   V   S   L   F   D   L   F   S   S   I   L
181/61                                                              211/71
AAT GGA AAG TTG GAT CCG TCG AAC TTT TCT TCA GAT TCA AGT GCT GCT ATT TTG ATT GAA
N   G   K   L   D   P   S   N   F   S   S   D   S   S   A   A   I   L   I   E
241/81                                                              271/91
AAT CGT GAG ATT TTA ATG ATC TTA ACA ACT GCT ATT GCT GTT TTT ATC GGT TGT GGT TTT
N   R   E   I   L   M   I   L   T   T   A   I   A   V   F   I   G   C   G   F
301/101                                                             331/111
CTC TAC GTT TGG AGA AGA TCT TCA AAT AAG TCG AGT AAA ATT GTT GAA ACT CAG AAA TTG
L   Y   V   W   R   R   S   S   N   K   S   S   K   I   V   E   T   Q   K   L
361/121                                                             391/131
ATC GTT GAA AAG GAA CCA GAA CCT GAA GTT GAT GAT GGA AAG AAG AAG GTT ACT ATC TTC
I   V   E   K   E   P   E   P   E   V   D   D   G   K   K   K   V   T   I   F
421/141                                                             451/151
TTT GGT ACT CAA ACT GGT ACA GCT GAA GGA TTC GCA AAG GCA CTT GCT GAA GAA GCA AAA
F   G   T   Q   T   G   T   A   E   G   F   A   K   A   L   A   E   E   A   K
481/161                                                             511/171
GCA AGA TAT GAA AAG GCA ATC TTT AAA GTG ATT GAT CTG GAT GAT TAC GGA GCA GAT GAT
A   R   Y   E   K   A   I   F   K   V   I   D   L   D   D   Y   G   A   D   D
541/181                                                             571/191
GAT GAA TTC GAA GAG AAA TTG AAA AAG GAA ACT ATA GCT CTT TTC TTT TTG GCT ACC TAT
D   E   F   E   E   K   L   K   K   E   T   I   A   L   F   F   L   A   T   Y
601/201                                                             631/211
GGA GAT GGT GAA CCT ACA GAT AAT GCT GCA AGA TTT TAT AAA TGG TTC ACA GAG GGA GAG
G   D   G   E   P   T   D   N   A   A   R   F   Y   K   W   F   T   E   G   E
661/221                                                             691/231
AGG GAA ATG TGG CTC CAG AAT CTT CAA TTT GGT GTC TTC GGT CTA GGC AAT AGA CAG TAT
R   E   M   W   L   Q   N   L   Q   F   G   V   F   G   L   G   N   R   Q   Y
721/241                                                             751/251
GAG CAT TTC AAT AAG GTG GCA AAG GAG GTG GAC GAG ATA CTC ACT GAA CAG GGT GGG AAG
E   H   F   N   K   V   A   K   E   V   D   E   I   L   T   E   Q   G   G   K
781/261                                                             811/271
CGT ATT GTT CCC GTG GGT CTA GGA GAT GAT GAT CAA TGC ATA GAA GAT GAT TTC ACT GCG
R   I   V   P   V   G   L   G   D   D   D   Q   C   I   E   D   D   F   T   A
841/281                                                             871/291
TGG CGG GAG TTG GTA TGG CCT GAA TTG GAT CAG TTG CTC CTT GAT GAA AGT GAT AAA ACA
W   R   E   L   V   W   P   E   L   D   Q   L   L   L   D   E   S   D   K   T
901/301                                                             931/311
TCT GTT TCT ACT CCT TAC ACT GCC ATC GTA CCA GAA TAC AGG GTA GTA TTC CAT GAT GCT
S   V   S   T   P   Y   T   A   I   V   P   E   Y   R   V   V   F   H   D   A
961/321                                                             991/331
ACT GAT GCA TCA CTA CAA GAC AAA AAC TGG AGC AAT GCA AAT GGC TAC ACT GTT TAC GAC
T   D   A   S   L   Q   D   K   N   W   S   N   A   N   G   Y   T   V   Y   D
1021/341                                                            1051/351
```

Figure 9b.

```
GTT CAA CAC CCA TGC AGA GCC AAT GTC GTT GTA AAG AAG GAG CTT CAC ACT CCA GTA TCT
V  Q  H  P  C  R  A  N  V  V  V  K  K  E  L  H  T  P  V  S
1081/361                                    1111/371
GAT CGT TCT TGT ATT CAT CTG GAA TTT GAC ATT TCT GGC ACT GGG CTC ACG TAT GAA ACA
D  R  S  C  I  H  L  E  F  D  I  S  G  T  G  L  T  Y  E  T
1141/381                                    1171/391
GGA GAC CAT GTC GGT GTT TAC TCT GAG AAT TGT GTT GAA GTT GTC GAG GAA GCA GAG AGG
G  D  H  V  G  V  Y  S  E  N  C  V  E  V  V  E  E  A  E  R
1201/401                                    1231/411
CTA TTG GGT TAC TCA TCA GAC ACC GTT TTT TCA ATC CAT GTC GAT AAA GAG GAC GGC TCA
L  L  G  Y  S  S  D  T  V  F  S  I  H  V  D  K  E  D  G  S
1261/421                                    1291/431
CCC ATT AGT GGA AGC GCT CTA GCT CCT CCT TTT CCA ACT CCC TGC ACT CTA AGA ACA GCA
P  I  S  G  S  A  L  A  P  P  F  P  T  P  C  T  L  R  T  A
1321/441                                    1351/451
CTA ACA CGA TAC GCT GAT CTG TTG AAT TCT CCC AAG AAG GCT GCT CTG CAT GCT TTG GCT
L  T  R  Y  A  D  L  L  N  S  P  K  K  A  A  L  H  A  L  A
1381/461                                    1411/471
GCT TAT GCA TCC GAT CCA AAG GAA GCG GAG CGA CTA AGG TAT CTT GCG TCT CCT GCT GGG
A  Y  A  S  D  P  K  E  A  E  R  L  R  Y  L  A  S  P  A  G
1441/481                                    1471/491
AAG GAC GAA TAC GCC CAG TGG ATA GTA GCT AGT CAG AGA AGT CTG CTA GTG GTC ATG GCT
K  D  E  Y  A  Q  W  I  V  A  S  Q  R  S  L  L  V  V  M  A
1501/501                                    1531/511
GAA TTC CCA TCA GCA AAG GCT CCA ATT GGG GTT TTC TTT GCA GCA GTA GCT CCT CGC TTG
E  F  P  S  A  K  A  P  I  G  V  F  F  A  A  V  A  P  R  L
1561/521                                    1591/531
CTG CCA AGA TAC TAT TCT ATT TCA TCT TCC AAT AGG ATG GTA CCA TCT AGG ATT CAT GTC
L  P  R  Y  Y  S  I  S  S  S  N  R  M  V  P  S  R  I  H  V
1621/541                                    1651/551
ACA TGT GCA TTG GTG CAT GAA AAA ACA CCG GCA GGT CGG GTT CAC AAA GGA GTG TGT TCA
T  C  A  L  V  H  E  K  T  P  A  G  R  V  H  K  G  V  C  S
1681/561                                    1711/571
ACC TGG ATG AAG AAT TCT GTG TCT TTG GAA GAA AAC CAT GAT TGC AGC AGC TGG GCA CCA
T  W  M  K  N  S  V  S  L  E  E  N  H  D  C  S  S  W  A  P
1741/581                                    1771/591
ATC TTT GTC AGG CAA TCC AAC TTC AAA CTT CCT GCT GAT TCT ACA GTA CCA ATT ATA ATG
I  F  V  R  Q  S  N  F  K  L  P  A  D  S  T  V  P  I  I  M
1801/601                                    1831/611
ATT GGT CCT GGG ACT GGA TTA GCT CCC TTT AGG GGA TTC ATG CAG GAG CGA TTA GCT CTG
I  G  P  G  T  G  L  A  P  F  R  G  F  M  Q  E  R  L  A  L
1861/621                                    1891/631
AAG AAT TCT GGT GTA GAA TTG GGA CCC GCT ATC CTC TTC TTT GGA TGC AGA AAC AGA CAG
K  N  S  G  V  E  L  G  P  A  I  L  F  F  G  C  R  N  R  Q
1921/641                                    1951/651
ATG GAT TAC ATA TAT GAA GAG GAG CTA AAC AAC TTT GTG AAA GAG GGA GCT ATC TCC GAA
M  D  Y  I  Y  E  E  E  L  N  N  F  V  K  E  G  A  I  S  E
1981/661                                    2011/671
GTT GTT GTT GCT TTC TCA CGT GAG GGA GCT ACC AAG GAA TAC GTA CAA CAT AAA ATG GCG
V  V  V  A  F  S  R  E  G  A  T  K  E  Y  V  Q  H  K  M  A
2041/681                                    2071/691
```

Figure 9b (cont.).

```
GAG AAG GCT TCC TAC ATC TGG GAA ATG ATC TCT CAA GGT GCT TAT CTT TAT GTA TGT GGT
E   K   A   S   Y   I   W   E   M   I   S   Q   G   A   Y   L   Y   V   C   G
2101/701                                2131/711
GAT GCC AAG GGC ATG GCT AGA GAC GTA CAT CGA ACT CTC CAC ACC ATT GCC CAG GAA CAG
D   A   K   G   M   A   R   D   V   H   R   T   L   H   T   I   A   Q   E   Q
2161/721                                2191/731
GGA TCT TTG GAC AAC TCG AAG ACC GAA AGC TTG GTG AAG AAT CTA CAG ATG GAT GGA AGG
G   S   L   D   N   S   K   T   E   S   L   V   K   N   L   Q   M   D   G   R
2221/741                                2251/751
TAT CTA CGT GAT GTG TGG TGA TTG ATT TTT TCA GCA CGG TTA CAA TCT AGC TTC ATC AAA
Y   L   R   D   V   W   *   L   I   F   S   A   R   L   Q   S   S   F   I   K
2281/761                                2311/771
GAA CGC GCT TGA GAA GCA TAA ATC TTA GTT GCA GAG ATG TTG ATT TCA GAA GAA ATG CTT
E   R   A   *   E   A   *   I   L   V   A   E   M   L   I   S   E   E   M   L
2341/781                                2371/791
TAT ATA CTT GAG GTA GCG GAC ATT AAT CCT TTT CTC TCT CTC TAA ACT GTT AAT CCT GTA
Y   I   L   E   V   A   D   I   N   P   F   L   S   L   *   T   V   N   P   V
2401/801                                2431/811
AAA AAG GGA TTG CTG TTT GTG TTT GCT CGC AAT CAA TTA AGT TAT ATT CTT TGG TCT ATG
K   K   G   L   L   F   V   F   A   R   N   Q   L   S   Y   I   L   W   S   M
2461/821                                2491/831
GCA TTC GTT AGA CAA ATA TAT TAA CGA GTT TGT CCG TTA TAT ATG ACA TAT GAA ACA AAA
A   F   V   R   Q   I   Y   *   R   V   C   P   L   Y   M   T   Y   E   T   K
2521/841                                2551/851
GAA CTT CTG TTT GGA GGA AGA GAA AAA AAA AAA AAA AA
E   L   L   F   G   G   R   E   K   K   K   K
```

Figure 9b (cont.).

```
 1 AAGCTTCAGAGTCTCTGCTAATT ATG GGT TCG AAT AAT TTA GCT AAT TCG ATT GAA TCG ATG TTA  65
 1                         M  G  S  N  N  L  A  N  S  I  E  S  M  L                14

66 GGA ATA TCA ATA GGA TCA GAA TAT ATT TCT GAC CCA ATT TTC ATT ATG GTC ACA ACT GTA 125
15 G  I  S  I  G  S  E  Y  I  S  D  P  I  F  I  M  V  T  T  V                    34

126 GCT TCA ATG CTG ATT GGA TTT GGT TTC TTC GCA TGT ATG AAA TCT TCG TCT TCT CAA TCA 185
35 A  S  M  L  I  G  F  G  F  F  A  C  M  K  S  S  S  S  Q  S                    54

186 AAA CCT ATT GAA ACT TAT AAA CCA ATA ATT GAT AAA GAA GAA GAG GAG ATT GAA GTT GAT 245
55 K  P  I  E  T  Y  K  P  I  I  D  K  E  E  E  E  I  E  V  D                    74

246 CCT GGT AAA ATT AAG CTC ACT ATA TTT TTT GGT ACT CAG ACT GGT ACT GCT GAA GGA TTT 305
75 P  G  K  I  K  L  T  I  F  F  G  T  Q  T  G  T  A  E  G  F                    94

306 GCT AAG GCA TTG GCA GAA GAA ATT AAG GCA AAG TAC AAG AAA GCA GTT GTT AAA GTA GTT 365
95 A  K  A  L  A  E  E  I  K  A  K  Y  K  K  A  V  V  K  V  V                   114

366 GAC CTG GAT GAC TAT GCA GCC GAG GAT GAT CAA TAT GAA GAG AAA TTA AAG AAA GAG TCT 425
115 D  L  D  D  Y  A  A  E  D  D  Q  Y  E  E  K  L  K  K  E  S                  134

426 TTG GTG TTT TTC ATG GTA GCC ACT TAT GGT GAT GGT GAG CCA ACT GAC AAT GCT GCG AGA 485
135 L  V  F  F  M  V  A  T  Y  G  D  G  E  P  T  D  N  A  A  R                  154

486 TTT TAC AAA TGG TTC ACT CAG GAA CAT GAA AGG GGA GAG TGG CTT CAG CAA CTA ACT TAT 545
155 F  Y  K  W  F  T  Q  E  H  E  R  G  E  W  L  Q  Q  L  T  Y                  174

546 GGT GTT TTT GGT TTG GGT AAC CGT CAA TAC GAG CAT TTC AAC AAG ATC GCG GTA GAT GTG 605
175 G  V  F  G  L  G  N  R  Q  Y  E  H  F  N  K  I  A  V  D  V                  194

606 GAT GAG CAA CTC GGT AAA CAA GGT GCA AAG CGC ATT GTT CAA GTG GGG CTC GGT GAC GAT 665
195 D  E  Q  L  G  K  Q  G  A  K  R  I  V  Q  V  G  L  G  D  D                  214

666 GAT CAA TGC ATT GAA GAT GAT TTT ACT GCT TGG CGA GAA TTG TTG TGG ACT GAA TTG GAT 725
215 D  Q  C  I  E  D  D  F  T  A  W  R  E  L  L  W  T  E  L  D                  234

726 CAG TTG CTC AAA GAT GAG GAT GCT GCT CCT TCA GTG GCT ACA CCG TAT ATT GCT ACT GTT 785
235 Q  L  L  K  D  E  D  A  A  P  S  V  A  T  P  Y  I  A  T  V                  254

786 CCT GAA TAC AGG GTA GTG ATT CAC GAA ACT ACG GTC GCG GCT CTG GAT GAT AAA CAC ATA 845
255 P  E  Y  R  V  V  I  H  E  T  T  V  A  A  L  D  D  K  H  I                  274

846 AAT ACT GCT AAC GGC GAT GTT GCA TTT GAT ATT CTC CAT CCT TGC AGA ACC ATT GTT GCT 905
275 N  T  A  N  G  D  V  A  F  D  I  L  H  P  C  R  T  I  V  A                  294

906 CAA CAA AGA GAG CTC CAC AAA CCC AAG TCT GAT AGA TCC TGT ATA CAT CTG GAG TTC GAC 965
295 Q  Q  R  E  L  H  K  P  K  S  D  R  S  C  I  H  L  E  F  D                  314

966 ATA TCA GGC TCT TCC CTT ACA TAT GAG ACT GGA GAT CAT GTT GGT GTT TAT GCT GAG AAC 1025
315 I  S  G  S  S  L  T  Y  E  T  G  D  H  V  G  V  Y  A  E  N                  334

1026 TGC GAT GAA ACT GTC GAG GAA GCA GGG AAG CTG TTG GGT CAA CCC CTG GAT TTG CTG TTT 1085
335 C  D  E  T  V  E  E  A  G  K  L  L  G  Q  P  L  D  L  L  F                  354

1086 TCA ATT CAC ACG GAT AAA GAA GAC GGG TCA CCC CAG GGA AGC TCA TTA CCA CCT CCT TTC 1145
355 S  I  H  T  D  K  E  D  G  S  P  Q  G  S  S  L  P  P  P  F                  374
```

Figure 10a

```
1146 CCA GGT CCT TGC ACC TTA CGA TCT GCC CTA GCA CGC TAT GCT GAT CTT TTG AAT CCT CCT 1205
 375 P   G   P   C   T   L   R   S   A   L   A   R   Y   A   D   L   L   N   P   P  394

1206 AGA AAG GCT TCT CTG ATT GCT CTG TCC GCT CAT GCA TCT GTA CCC AGT GAA GCA GAG AGA 1265
 395 R   K   A   S   L   I   A   L   S   A   H   A   S   V   P   S   E   A   E   R  414

1266 TTG CGC TTT TTG TCA TCA CCT CTG GGA AAG AAT GAG TAT TCA AAA TGG GTA GTT GGA AGT 1325
 415 L   R   F   L   S   S   P   L   G   K   N   E   Y   S   K   W   V   V   G   S  434

1326 CAG AGG AGT CTT TTG GAG ATC ATG GCC GAG TTT CCA TCA GCA AAA CCC CCT CTT GGT GTT 1385
 435 Q   R   S   L   L   E   I   M   A   E   F   P   S   A   K   P   P   L   G   V  454

1386 TTC TTT GCT GCA GTA GCC CCT CGC TTA CCG CCT CGA TAC TAT TCT ATC TCA TCC TCT CCT 1445
 455 F   F   A   A   V   A   P   R   L   P   P   R   Y   Y   S   I   S   S   S   P  474

1446 AAG TTT GCT CCC TCA AGA ATT CAT GTG ACG TGT GCT TTA GTA TAT GGT CAA AGC CCT ACC 1505
 475 K   F   A   P   S   R   I   H   V   T   C   A   L   V   Y   G   Q   S   P   T  494

1506 GGA AGG GTT CAC CGA GGA GTG TGT TCG ACA TGG ATG AAG CAT GCA GTT CCT CAG GAT AGC 1565
 495 G   R   V   H   R   G   V   C   S   T   W   M   K   H   A   V   P   Q   D   S  514

1566 TGG GCT CCT ATT TTT GTT CGA ACG TCA AAC TTC AAG TTA CCA GCT GAC CCC TCA ACT CCA 1625
 515 W   A   P   I   F   V   R   T   S   N   F   K   L   P   A   D   P   S   T   P  534

1626 ATT ATC ATG GTG GGA CCT GGT ACA GGG TTA GCT CCT TTC AGA GGA TTT CTG CAG GAA AGA 1685
 535 I   I   M   V   G   P   G   T   G   L   A   P   F   R   G   F   L   Q   E   R  554

1686 ATG GCC CTC AAG GAA AAT GGT GCT CAA CTT GGC CCA GCA GTG CTC TTT TTC GGA TGT AGG 1745
 555 M   A   L   K   E   N   G   A   Q   L   G   P   A   V   L   F   F   G   C   R  574

1746 AAT CGT AAT ATG GAC TTC ATT TAT GAA GAC GAA CTA AAC AAC TTC GTG GAA CGA GGA GTA 1805
 575 N   R   N   M   D   F   I   Y   E   D   E   L   N   N   F   V   E   R   G   V  594

1806 ATT TCG GAG CTA GTT ATT GCC TTT TCA CGT GAA GGG GAA AAG AAG GAA TAT GTT CAA CAT 1865
 595 I   S   E   L   V   I   A   F   S   R   E   G   E   K   K   E   Y   V   Q   H  614

1866 AAG ATG ATG GAG AAA GCA ACG GAT GTA TGG AAT GTG ATA TCA GGG GAC GGT TAT CTC TAT 1925
 615 K   M   M   E   K   A   T   D   V   W   N   V   I   S   G   D   G   Y   L   Y  634

1926 GTG TGT GGT GAT GCC AAG GGA ATG GCC AGA GAT GTC CAT CGC ACG TTG CAT ACC ATT GCC 1985
 635 V   C   G   D   A   K   G   M   A   R   D   V   H   R   T   L   H   T   I   A  654

1986 CAA GAA CAG GGA CCC ATG GAA TCA TCT GCT GCC GAA GCT GCA GTA AAG AAA CTC CAA GTT 2045
 655 Q   E   Q   G   P   M   E   S   S   A   A   E   A   A   V   K   K   L   Q   V  674

2046 GAA GAA CGA TAT CTA AGA GAT GTC TGG TGA TCGA ATG TAG CTTGCCAAtcactag            2100
 675 E   E   R   Y   L   R   D   V   W   *       M   *                              2
```

Figure 10a (cont.).

```
   1 tgcagcccgggggatccgcccT ATG GAA CAA ACT GCG GTT AAA GTC TCT TTG TTT GAT CTA TTT   64
   1                        M  E  Q  T  A  V  K  V  S  L  F  D  L  F                14

  65 TCT TCG ATA CTT AAT GGA AAG TTG GAT CCG TCG AAC TTT TCT TCA GAT TCA AGT GCT GCT  124
  15 S  S  I  L  N  G  K  L  D  P  S  N  F  S  S  D  S  S  A  A                    34

 125 ATT TTG ATT GAA AAT CGT GAG ATT TTA ATG ATC TTA ACA ACT GCT ATT GCT GTT TTT ATC  184
  35 I  L  I  E  N  R  E  I  L  M  I  L  T  T  A  I  A  V  F  I                    54

 185 GGT TGT GGT TTT CTC TAC GTT TGG AGA AGA TCT TCA AAT AAG TCG AGT AAA ATT GTT GAA  244
  55 G  C  G  F  L  Y  V  W  R  R  S  S  N  K  S  S  K  I  V  E                    74

 245 ACT CAG AAA TTG ATC GTT GAA AAG GAA CCA GAA CCT GAA GTT GAT GAT GGA AAG AAG AAG  304
  75 T  Q  K  L  I  V  E  K  E  P  E  P  E  V  D  D  G  K  K  K                    94

 305 GTT ACT ATC TTC TTT GGT ACT CAA ACT GGT ACA GCT GAA GGA TTC GCA AAG GCA CTT GCT  364
  95 V  T  I  F  F  G  T  Q  T  G  T  A  E  G  F  A  K  A  L  A                   114

 365 GAA GAA GCA AAA GCA AGA TAT GAA AAG GCA ATC TTT AAA GTG ATT GAT CTG GAT GAT TAC  424
 115 E  E  A  K  A  R  Y  E  K  A  I  F  K  V  I  D  L  D  D  Y                   134

 425 GGA GCA GAT GAT GAT GAA TTC GAA GAG AAA TTG AAA AAG GAA ACT ATA GCT CTT TTC TTT  484
 135 G  A  D  D  D  E  F  E  E  K  L  K  K  E  T  I  A  L  F  F                   154

 485 TTG GCT ACC TAT GGA GAT GGT GAA CCT ACA GAT AAT GCT GCA AGA TTT TAT AAA TGG TTC  544
 155 L  A  T  Y  G  D  G  E  P  T  D  N  A  A  R  F  Y  K  W  F                   174

 545 ACA GAG GGA GAG AGG GAA ATG TGG CTC CAG AAT CTT CAA TTT GGT GTC TTC GGT CTA GGC  604
 175 T  E  G  E  R  E  M  W  L  Q  N  L  Q  F  G  V  F  G  L  G                   194

 605 AAT AGA CAG TAT GAG CAT TTC AAT AAG GTG GCA AAG GAG GTG GAC GAG ATA CTC ACT GAA  664
 195 N  R  Q  Y  E  H  F  N  K  V  A  K  E  V  D  E  I  L  T  E                   214

 665 CAG GGT GGG AAG CGT ATT GTT CCC GTG GGT CTA GGA GAT GAT GAT CAA TGC ATA GAA GAT  724
 215 Q  G  G  K  R  I  V  P  V  G  L  G  D  D  D  Q  C  I  E  D                   234

 725 GAT TTC ACT GCG TGG CGG GAG TTG GTA TGG CCT GAA TTG GAT CAG TTG CTC CTT GAT GAA  784
 235 D  F  T  A  W  R  E  L  V  W  P  E  L  D  Q  L  L  L  D  E                   254

 785 AGT GAT AAA ACA TCT GTT TCT ACT CCT TAC ACT GCC ATC GTA CCA GAA TAC AGG GTA GTA  844
 255 S  D  K  T  S  V  S  T  P  Y  T  A  I  V  P  E  Y  R  V  V                   274

 845 TTC CAT GAT GCT ACT GAT GCA TCA CTA CAA GAC AAA AAC TGG AGC AAT GCA AAT GGC TAC  904
 275 F  H  D  A  T  D  A  S  L  Q  D  K  N  W  S  N  A  N  G  Y                   294

 905 ACT GTT TAC GAC GTT CAA CAC CCA TGC AGA GCC AAT GTC GTT GTA AAG AAG GAG CTT CAC  964
 295 T  V  Y  D  V  Q  H  P  C  R  A  N  V  V  V  K  K  E  L  H                   314

 965 ACT CCA GTA TCT GAT CGT TCT TGT ATT CAT CTG GAA TTT GAC ATT TCT GGC ACT GGG CTC 1024
 315 T  P  V  S  D  R  S  C  I  H  L  E  F  D  I  S  G  T  G  L                   334

1025 ACG TAT GAA ACA GGA GAC CAT GTC GGT GTT TAC TCT GAG AAT TGT GTT GAA GTT GTC GAG 1084
 335 T  Y  E  T  G  D  H  V  G  V  Y  S  E  N  C  V  E  V  V  E                   354

1085 GAA GCA GAG AGG CTA TTG GGT TAC TCA TCA GAC ACC GTT TTT TCA ATC CAT GTC GAT AAA 1144
 355 E  A  E  R  L  L  G  Y  S  S  D  T  V  F  S  I  H  V  D  K                   374
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Figure 10b

```
1145 GAG GAC GGC TCA CCC ATT AGT GGA AGC GCT CTA GCT CCT CCT TTT CCA ACT CCC TGC ACT 1204
 375 E   D   G   S   P   I   S   G   S   A   L   A   P   P   F   P   T   P   C   T   394

1205 CTA AGA ACA GCA CTA ACA CGA TAC GCT GAT CTG TTG AAT TCT CCC AAG AAG GCT GCT CTG 1264
 395 L   R   T   A   L   T   R   Y   A   D   L   L   N   S   P   K   K   A   A   L   414

1265 CAT GCT TTG GCT GCT TAT GCA TCC GAT CCA AAG GAA GCG GAG CGA CTA AGG TAT CTT GCG 1324
 415 H   A   L   A   A   Y   A   S   D   P   K   E   A   E   R   L   R   Y   L   A   434

1325 TCT CCT GCT GGG AAG GAC GAA TAC GCC CAG TGG ATA GTA GCT AGT CAG AGA AGT CTG CTA 1384
 435 S   P   A   G   K   D   E   Y   A   Q   W   I   V   A   S   Q   R   S   L   L   454

1385 GTG GTC ATG GCT GAA TTC CCA TCA GCA AAG GCT CCA ATT GGG GTT TTC TTT GCA GCA GTA 1444
 455 V   V   M   A   E   F   P   S   A   K   A   P   I   G   V   F   F   A   A   V   474

1445 GCT CCT CGC TTG CTG CCA AGA TAC TAT TCT ATT TCA TCT TCC AAT AGG ATG GTA CCA TCT 1504
 475 A   P   R   L   L   P   R   Y   Y   S   I   S   S   S   N   R   M   V   P   S   494

1505 AGG ATT CAT GTC ACA TGT GCA TTG GTG CAT GAA AAA ACA CCG GCA GGT CGG GTT CAC AAA 1564
 495 R   I   H   V   T   C   A   L   V   H   E   K   T   P   A   G   R   V   H   K   514

1565 GGA GTG TGT TCA ACC TGG ATG AAG AAT TCT GTG TCT TTG GAA GAA AAC CAT GAT TGC AGC 1624
 515 G   V   C   S   T   W   M   K   N   S   V   S   L   E   E   N   H   D   C   S   534

1625 AGC TGG GCA CCA ATC TTT GTC AGG CAA TCC AAC TTC AAA CTT CCT GCT GAT TCT ACA GTA 1684
 535 S   W   A   P   I   F   V   R   Q   S   N   F   K   L   P   A   D   S   T   V   554

1685 CCA ATT ATA ATG ATT GGT CCT GGG ACT GGA TTA GCT CCC TTT AGG GGA TTC ATG CAG GAG 1744
 555 P   I   I   M   I   G   P   G   T   G   L   A   P   F   R   G   F   M   Q   E   574

1745 CGA TTA GCT CTG AAG AAT TCT GGT GTA GAA TTG GGA CCC GCT ATC CTC TTC TTT GGA TGC 1804
 575 R   L   A   L   K   N   S   G   V   E   L   G   P   A   I   L   F   F   G   C   594

1805 AGA AAC AGA CAG ATG GAT TAC ATA TAT GAA GAG GAG CTA AAC AAC TTT GTG AAA GAG GGA 1864
 595 R   N   R   Q   M   D   Y   I   Y   E   E   E   L   N   N   F   V   K   E   G   614

1865 GCT ATC TCC GAA GTT GTT GTT GCT TTC TCA CGT GAG GGA GCT ACC AAG GAA TAC GTA CAA 1924
 615 A   I   S   E   V   V   V   A   F   S   R   E   G   A   T   K   E   Y   V   Q   634

1925 CAT AAA ATG GCG GAG AAG GCT TCC TAC ATC TGG GAA ATG ATC TCT CAA GGT GCT TAT CTT 1984
 635 H   K   M   A   E   K   A   S   Y   I   W   E   M   I   S   Q   G   A   Y   L   654

1985 TAT GTA TGT GGT GAT GCC AAG GGC ATG GCT AGA GAC GTA CAT CGA ACT CTC CAC ACC ATT 2044
 655 Y   V   C   G   D   A   K   G   M   A   R   D   V   H   R   T   L   H   T   I   674

2045 GCC CAG GAA CAG GGA TCT TTG GAC AAC TCG AAG ACC GAA AGC TTG GTG AAG AAT CTA CAG 2104
 675 A   Q   E   Q   G   S   L   D   N   S   K   T   E   S   L   V   K   N   L   Q   694

2105 ATG GAT GGA AGG TAT CTA CGT GAT GTG TGG TGA TTGgggctagagcggcc                   2154
 695 M   D   G   R   Y   L   R   D   V   W   *                                     705
```

Figure 10b (cont.).

DOCKET NO. J&J-1673

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled CYTOCHROME P450 REDUCTASES FROM POPPY PLANTS, the specification of which

(check one) ☐ is attached hereto.

☒ was filed on February 29, 2000 as

Application Serial No. 09/486,757

and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s):

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
Australia	PO 8872	August 29, 1997	☒ YES ☐ NO
	PCT/AU98/00705	August 28, 1998	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(Application Number) (Filing Date)

(Application Number) (Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No. Filing Date Status

Application Serial No. Filing Date Status

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith as well as to file equivalent patent applications in countries foreign to the United States including the filing of international patent applications in accordance with the Patent Cooperation Treaty: Audley A. Ciamporcero, Jr. (Reg. #26,051), Steven P. Berman (Reg. #24,772), Andrea L. Colby (Reg. #30,194), Ralph R. Palo (Reg. #29,486), Michael Stark (Reg. #32,495), and John W.

Harbour (Reg. #31,365) One Johnson & Johnson Plaza, New Brunswick, NJ 08933.

Address all telephone calls to John W. Harbour at telephone no. (732) 524-2169.

Address all correspondence to Audley A. Ciamporcero, Jr., One Johnson & Johnson Plaza, New Brunswick, NJ 08933-7003.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventor's Signature: Toni M. Kutchan
Full Name of Sole or First Inventor: Toni M. Kutchan
Date: 14 Jun 2000 DEX

Citizenship: USA
Residence: Carl-von-Ossietzky-Str. 11, 06114 Halle, Germany
Post Office Address: Same as the above

Inventor's Signature: Meinhart H. Zenk
Full Name of Second Joint Inventor, If Any: Meinhart H. Zenk
Date: 14 June 2000 DEX

Citizenship: DE
Residence: Carl-von-Ossietzky-Str. 11, 06114 Halle, Germany
Post Office Address: Same as the above

Inventor's Signature:
Full Name of Third Joint Inventor, If Any: David G. Atkins
Date: 30 June 2000

Citizenship: AU
Residence: 46 W 60th St., Apt. 17A, New York, NY 10023, USA
Post Office Address: Same as the above

Inventor's Signature: _____

Full Name of Third Joint Inventor, If Any: Anthony J. Fist

Date: _____

Citizenship: AU
Residence: 36 Beech Road, Norwood, Tas 7250 Australia
Post Office Address: Same as the above

(Supply similar information and signature for fourth and subsequent joint inventors.)

DOCKET NO. J&J-1673

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled CYTOCHROME P450 REDUCTASES FROM POPPY PLANTS, the specification of which

(check one) ☐ is attached hereto.

☒ was filed on February 29, 2000 as

Application Serial No. 09/486,757

and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s):

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
Australia	8872	August 29, 1997	☒ YES ☐ NO
	PCT/AU98/00705	August 28, 1998	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(Application Number) (Filing Date)

(Application Number) (Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No. Filing Date Status

Application Serial No. Filing Date Status

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith as well as to file equivalent patent applications in countries foreign to the United States including the filing of international patent applications in accordance with the Patent Cooperation Treaty: Audley A. Ciamporcero, Jr. (Reg. #26,051), Steven P. Berman (Reg. #24,772), Andrea L. Colby (Reg. #30,194), Ralph R. Palo (Reg. #29,486), Michael Stark (Reg. #32,495), and John W. Harbour (Reg. #31,365) One Johnson & Johnson Plaza, New Brunswick, NJ 08933.

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Inventor's Signature: _____

Full Name of Sole or First Inventor: Toni M. Kutchan

Date: _____

Citizenship: USA

Residence: Pfeiveftistrasse 17, Munich, 81243 Germany

Post Office Address: Same as the above

Inventor's Signature: _____

Full Name of Second Joint Inventor, If Any: Meinhart H. Zenk

Date: _____

Citizenship: DE

Residence: Pfeiveftlstrasse 17, Munich, 81243, Germany

Post Office Address: Same as the above

Inventor's Signature: _____

Full Name of Third Joint Inventor, If Any: David G. Atkins

Date: _____

Citizenship: ~~DE~~

Residence: ~~82 Womerah Avenue, Darlinghurst, NSW 2010, Australia~~ (Refer to Document with Original Signature)

Post Office Address: Same as the above

Inventor's Signature:

Full Name of ~~Third~~ Fourth Joint Inventor, If Any: Anthony J. Fist

Date: 8 June 2000

Citizenship: AU

Residence: 36 Beech Road, Norwood, Tas 7250, Australia

Post Office Address: Same as the above AUS

(Supply similar information and signature for fourth and subsequent joint inventors.)